Elastase activity on sputum neutrophils correlates with severity of lung disease in cystic fibrosis

A. Susanne Dittrich[1,2,3,5], Iris Kühbandner[1,2,3], Stefanie Gehrig[1,2,4,5], Verena Rickert-Zacharias[1,2,4,5], Matthew Twigg[6], Sabine Wege[2,3], Clifford C. Taggart[6], Felix Herth[2,3], Carsten Schultz[2,4,5] and Marcus A. Mall[1,2,5]

[1]Department of Translational Pulmonology and Division of Pediatric Pulmonology & Allergy and Cystic Fibrosis Center, University of Heidelberg, Heidelberg, Germany

[2]Translational Lung Research Center Heidelberg (TLRC), Member of the German Center for Lung Research (DZL), Heidelberg, Germany

[3]Department of Pneumology and Critical Care Medicine, Thoraxklinik at the University Hospital Heidelberg, Heidelberg, Germany

[4]Cell Biology and Biophysics Unit, European Molecular Biology Laboratory, Heidelberg, Germany

[5]Molecular Medicine Partnership Unit (MMPU), University of Heidelberg and European Molecular Biology Laboratory, Heidelberg, Germany

[6]Centre for Experimental Medicine, Queen’s University Belfast, Belfast, United Kingdom

Corresponding author:

Marcus A. Mall, Department of Translational Pulmonology and Division of Pediatric Pulmonology & Allergy and Cystic Fibrosis Center, Translational Lung Research Center Heidelberg (TLRC), University of Heidelberg, Im Neuenheimer Feld 156, 69120 Heidelberg, Germany. Phone: +49-6221-568840; Fax: +49-6221-568806; Email: Marcus.Mall@med.uni-heidelberg.de.

Author’s contributions: Conception and design of the study: A.S.D, C.S., M.A.M; Acquisition, analysis and interpretation of data: A.S.D., I.K., S.G., V.R.Z., C.C.T., M.T., S.W.,

F.H., C.S., M.A.M.; Writing the manuscript or revising it critically for important intellectual content: A.S.D., C.C.T., F.H., C.S., M.A.M.

Sources of support:

Funded in part by the German Ministry for Education and Research (FKZ 82DZL00401 and FKZ 82DZL004A1 to M.A.M and C.S.), the European Commission (Seventh Framework Programme Project No. 603038 CFMatters to M.A.M) and the German Cystic Fibrosis Association Mukoviszidose e. V. (Project number 1605 to A.S.D.). A.S.D. was supported by a HRCMM (Heidelberg Research Center for Molecular Medicine) Career Development Fellowship.

Running title: Increased elastase activity on CF sputum neutrophils

Descriptor: 9.17 Cystic Fibrosis: Translational & Clinical Studies

Word count for manuscript body: 2772 words

AT A GLANCE COMMENTARY

Scientific knowledge on the subject

Neutrophil elastase (NE) is a key risk factor for the onset and progression of structural lung disease in patients with cystic fibrosis (CF). Recent studies identified increased NE activity on the surface of neutrophils from airways of mice with CF-like lung disease and patients with CF, even in the absence of free NE activity in airway samples. However, the relationship between increased NE activity on the surface of airway neutrophils and severity of lung disease in patients with CF remains unknown.

What this study adds to the field

This study investigated the relationship between NE activity on the surface of sputum neutrophils and severity of lung disease in adult patients with CF. We demonstrate that NE activity is increased on the surface of CF sputum neutrophils, even at low levels of free NE activity in cell-free sputum supernatants. Further, cell surface-bound NE activity correlated inversely with FEV_1 % predicted and FRCpleth % predicted as outcome measures of airflow limitation and air trapping. These results suggest that surface-bound NE activity may contribute to severity of lung disease and serve as a novel biomarker in patients with CF.

This article has an online data supplement, which is accessible from this issue's table of content online at www.atsjournals.org.

ABSTRACT

Rationale: Neutrophil elastase (NE) is a key risk factor for the onset and progression of cystic fibrosis (CF) lung disease. Recent studies identified increased NE activity on the surface of neutrophils from airways of mice with CF-like lung disease and patients with CF. However, the role of NE activity on neutrophil surfaces in CF lung disease remains unknown.

Objectives: To determine the relationship between surface-bound NE activity on sputum neutrophils and severity of lung disease in patients with CF.

Methods: Surface-bound NE activity was measured on sputum neutrophils from a prospective cohort of 35 patients with CF using novel lipidated and soluble Foerster resonance energy transfer (FRET) reporters and correlated with free NE activity, neutrophil counts, IL-8, MPO and antiproteases in cell-free sputum supernatants and with parameters of lung function.

Measurement and main results Surface-bound NE activity on sputum neutrophils was increased in CF compared to heathly controls (P<0.01) and correlated with free NE activity (P<0.05), but not with other inflammation markers in CF sputum Surface-bound and free NE activity correlated with FEV_1 % predicted (P<0.01 and P<0.05), but only surface-bound NE activity correlated with FRCpleth % predicted (P<0.01).

Conclusions: Surface-bound NE activity on airway neutrophils is increased and correlates with severity of lung disease independent of other markers of inflammation in patients with CF. Surface-bound NE activity, probably reflecting a freshly secreted pool of NE not inhibited by antiproteases, may play an important role in the pathogenesis and serve as novel biomarker of neutrophil activation in CF lung disease.

Abstract word count: 250 words

Keywords: airway inflammation; cystic fibrosis, neutrophilic airway disease, biomarker, protease

INTRODUCTION

Chronic neutrophilic airway inflammation is a hallmark of cystic fibrosis (CF) and increased activity of neutrophil elastase (NE), a major product of activated neutrophils, has been identified as a key risk factor for the onset and progression of bronchiectasis and lung function decline in patients with CF (1–3). Beyond structural damage of airway walls, NE has been implicated in the pathogenesis of mucus hypersecretion (4–6), airway inflammation (7–9), and impaired defenses against *Pseudomonas aeruginosa* infection (10–14). In addition, increased NE activity was shown to disable CF transmembrane conductance regulator (CFTR) Cl^- channels and activate epithelial Na^+ channels (ENaC), thus aggravating basic defects in anion secretion and Na^+ absorption in CF airways (15–17). These results from observational and experimental studies suggest NE activity in airway specimen such as sputum or bronchoalveolar lavage (BAL) fluid as a promising biomarker of neutrophilic inflammation in CF lung disease (2, 3). So far, measurements have focused on the detection of free NE activity in BAL and sputum supernatant (2, 3, 18–22). Since NE is a highly cationic molecule, a significant proportion of secreted NE is bound to the neutrophil membrane via electrostatic interactions (23–25). Using a novel Foerster resonance energy transfer (FRET)-based NE reporter assay (26), we recently demonstrated that NE activity is also increased on the surface of airway neutrophils in patients with CF (4). In addition, we showed that elevated surface-bound NE activity is implicated in airway inflammation, mucus hyper-secretion and structural lung damage in mice with CF-like lung disease, even in the context of moderate airway neutrophilia, where free NE is effectively inhibited by an intact antiprotease shield and no free NE activity is detectable in BAL fluid (4). However, the role of NE activity on neutrophil surfaces in CF lung disease remains unknown.

The aim of this study was, therefore, to determine the relationship between surface-bound NE activity on the cell membrane of airway neutrophils and the severity of lung disease in patients with CF. To achieve this goal, we collected sputum from a prospective cohort of 35 patients with CF and 8 healthy non-smokers and used FRET reporters to quantify surface-

bound NE activity on sputum neutrophils and free NE activitiy in sputum supernatants. Further, we determined sputum neutrophil counts and levels of interleukin-8 (IL-8), myeloperoxidase (MPO) and antiproteases such as α1-antitrypsin-NE complexes (AAT-NE) and secretory leukocyte protease inhibitor (SLPI) in sputum supernatant, performed paired pulmonary function testing, and correlated surface-bound NE activity with these markers of disease severity in patients with CF. Some of the results have been previously reported in the form of abstracts (27, 28).

METHODS

Study population

This study was approved by the ethics committee of the University of Heidelberg and informed written consent was obtained from all subjects. The diagnosis of CF was verified by established diagnostic criteria (29, 30). Spontaneously expectorated (n=36) or induced sputum (n=3) of sufficient quantity (total cell count of inflammatory cells >60,000) was collected from 35 clinically stable patients with CF during routine visits at the CF Center at the University Hospital Heidelberg. Patient characteristics are summarized in Table 1 and *CFTR* genotypes are provided in Table E1. Colonization with *Pseudomonas aeruginosa* was defined as negative when 0%, intermittent when ≤50% and chronic when >50% of airway cultures were positive in the previous twelve month. Airflow obstruction and pulmonary hyperinflation were detected by measurement of forced expiratory volume in one second (FEV_1) and plethysmographic functional residual capacity (FRCpleth) on the same day of sputum collection according to ATS/ERS guidelines (31, 32). As control group, we collected induced sputum from 8 age-matched, healthy, non-smoking volunteers (5 females and 3 males; median age 31.1 range 22.01-45.02 years) by hypertonic saline inhalation as previously described (4, 33). Briefly, the mouth was rinsed with water and 100 µg salbutamol was inhaled prior to 6% hypertonic saline. Sputum samples were directly collected in a specimen container, stored on ice immediately after production and processed within two

hours.

Sputum processing and measurements of free and surface-bound NE activity, cytokines and antiproteases

Sputum was separated from saliva and homogenized using 10% sputolysin (Calbiochem, Darmstadt, Germany). Sputum inflammatory cells were isolated and total immune cell counts were performed. Free NE activity was quantified in sputum supernatants using the FRET probe NEmo-1 and compared with activity levels determined by the chromogenic substrate MeO-Suc-AAPV-pNA (Sigma, St Louis, MO, USA) (1–3). Surface-bound NE activity on sputum neutrophils was measured using the lipidated FRET reporter NEmo-2 (4, 26) and determined from the ratio of donor to acceptor fluorescence (D/A ratio) as previously described and detailed in the online supplement (4). Levels of IL-8, MPO, SLPI and AAT-NE were measured using ELISA. Differential cell counts were performed on May-Grünwald-Giemsa stained cytospin preparations. For three CF and one control subject, differential cell count could not be determined because the cytospins were of poor quality. Values below the detection limit are reported as 0. Additional information is provided in the online supplement.

Statistical analysis

Data were analyzed with "R" (R Foundation for Statistical Computing, Vienna, Austria) or SigmaPlot (Systat Software GmbH, Erkrath, Germany) and are reported as median (interquartile range, 25–75th percentile). Statistical analysis was performed using Shapiro-wilk test, unpaired Student's t test, Wilcoxon rank sum test, one-way analysis of variance, Kruskal-Wallis test, Dunn's test or Dunn's test versus control as appropriate. Correlation analyses were performed using the Spearman rank order method. A *P* value less than 0.05 was considered statistically significant. In the case of multiple comparisons, individual *P* levels are indicated only if not rejected by the Bonferroni–Holm method.

RESULTS

NE activity is increased on the surface of sputum neutrophils in CF, even in patients with low levels of free NE activity in sputum supernatant

To determine surface-bound NE activity on airway neutrophils and its relationship with free NE activity in CF airway inflammation, we assessed prospectively collected sputum samples from 35 clinically stable adult patients with CF (Table 1) and 8 healthy non-smokers using highly sensitive NE-specific FRET reporters developed to quantify free (NEmo-1) and surface-bound (NEmo-2) NE activity (4, 26). As expected, absolute neutrophil counts (P<0.001) and percentage of neutrophils (P<0.001) in sputum were significantly increased in our cohort of adult patients with CF compared to healthy controls (Figures 1A and 1B). Free NE activity detected by NEmo-1 was significantly increased in CF compared to control sputum (P<0.01), was completely inhibited by the NE inhibitor sivelestat (Figure 1D), and showed a strong correlation with free NE activity detected by the established chromogenic substrate MeO-Suc-AAPV-pNA (r=0.91, P<0.001 and Figure E1). Similar to free NE activity, surface-bound NE activity was significantly increased on CF compared to control neutrophils (P<0.01) and completely inhibited by sivelestat (Figure 1C and 1E). Surface-bound and free NE activity showed a moderate correlation in CF samples (P<0.05, Figure 1F). Of note, grouping of CF samples according to the levels (quartiles) of free NE activity in sputum supernatant demonstrated that surface-bound NE activity was significantly increased on CF compared to control neutrophils, even in patients with low levels (≤25th percentile) of free NE activity (P<0.01; Figure 1G). Taken together, these results demonstrate that, in addition to free NE activity in airway secretions, surface-bound NE activity on airway neutrophils is increased and contributes to the protease burden in the lungs of patients with CF.

Relationship of surface-bound and free NE activity with markers of neutrophilic inflammation and antiproteases

Next, we determined the relationship of free and surface-bound NE activity in CF sputum with markers of neutrophilic inflammation including neutrophil counts, IL-8 and MPO, and

antiproteases that inhibit NE activity in the airway lumen. Free NE activity in CF sputum supernatants correlated with absolute neutrophil counts, IL-8 and MPO (Figures 2A, C and E). Surface-bound NE activity did not correlate with these markers of neutrophilic inflammation (Figures 2 B, D and F). Neither free nor surface-bound NE activity correlated with percentage of sputum neutrophils (r=0.11, *P*=0.53 and r=0.15, *P*=0.36). To investigate the relationship of free and surface-bound NE activity with antiproteases, we measured levels of SLPI and complexes of NE with α1-antitrypsin (AAT-NE), two major endogenous inhibitors of NE in the airways (34), in sputum supernatants. As expected from previous studies (10, 35), AAT-NE was significantly increased (*P*<0.001), whereas SLPI was decreased (*P*<0.001) in CF compared to control sputum (Table 2). Free, but not surface-bound NE activity correlated with AAT-NE (Figure 2G and H). Neither free, nor surface-bound NE activity were correlated with SLPI (Figure 2I and J). Collectively, these data indicate that free NE activity depends on the level of airway inflammation, as determined from the number of neutrophils, levels of IL-8 and AAT-NE in sputum, whereas surface-bound NE activity is increased on CF neutrophils independent of these indices of neutrophilic airway inflammation.

Surface-bound NE activity on sputum neutrophils correlates with airflow obstruction and air trapping

To determine the clinical relevance of increased NE activity on the surface of CF neutrophils, we correlated free and surface-bound NE activity with FEV_1 and (FRCpleth) as lung function parameters of airflow obstruction and air trapping. As expected from previous studies (3, 19, 22, 36), free NE activity in CF sputum showed a negative correlation with FEV_1 % predicted (*P*<0.05) in our cohort of patients with CF (Figure 3A), whereas no relationship was found between free NE and FRCpleth % predicted (Figure 3C). Surface-bound NE activity on CF sputum neutrophils was also inversely correlated with FEV_1 % predicted (*P*<0.01, Figure 3B). In addition, surface-bound NE activity was directly correlated with FRC % predicted in patients with CF (*P*<0.01, Figure 3D). These results show that the level of surface-bound NE

activity is related to the severity of airflow obstruction and air trapping in adult patients with CF.

DISCUSSION

The mucostatic environment in CF lung disease sustains a destructive cascade of oxidative stress, ineffective host defense and chronic infection, resulting in a massive recruitment of neutrophils to the lungs (37). During homing to the CF airways, viable neutrophils undergo activation and extensively mobilize NE-rich granula to the cell surface (38, 39). *In vitro* studies demonstrated that activated blood neutrophils express 6-fold more NE on the cell surface than is freely released, whereas unstimulaed neutrophils express only minimal amounts of NE on the cell surface (24). NE binds charge-dependently to low affinity, high volume binding sites on the neutrophil membrane (25) and surface-bound NE has a similar spectrum of catalytic activity and efficiency as free NE (24, 25, 40). The clinical relevance of NE activity on the cell surface of intact airway neutrophils in CF lung disease, however, remains unknown. Thus, we conducted this prospective, cross-sectional study in 35 patients with CF covering a wide spectrum of disease severity to compare the role of surface-bound with free NE activity in advanced CF lung disease. Our work shows for the first time that surface-bound NE activity is associated with severity of CF lung disease.

Preclinical studies utilizing βENaC-overexpressing mice, an established model of CF-like lung disease, provided first insights into the pathophysiologic role of surface-bound NE activity in the context of early-onset airway mucus plugging, spontaneous bacterial infection and chronic airway inflammation (4, 41–44). Similar to mild lung disease in infants and young children with CF, this mouse model exhibits a moderate airway neutrophila with 5-30 % neutrophils in BAL fluid (1, 41, 44). Genetic deletion of NE demonstrated that a lack of NE significantly reduces airway inflammation, mucus hypersecretion and structural lung damage in βENaC-overexpressing mice. However, no free NE activity was detectable in the cell-free

supernatants of BAL fluid from βENaC-overexpressing mice, whereas surface-bound NE activity on BAL neutrophils was constantly increased. This was likely due to an intact antiprotease shield, as BAL supernatants could inhibit activity of purified NE in a dose-dependent manner (4). So far, the massive influx, necrosis, cell lysis and impaired clearance of neutrophils has been considered as the main mechanism leading to NE burden in advanced CF lung disease (37). In our cohort, sputum neutrophil counts were markedly elevated, consistent with reported neutrophil numbers in sputum from adult patients with CF (4, 20). However, we found that surface-bound NE activity was increased on sputum neutrophils, even when free NE activity was low. Further, free but not surface-bound NE activity correlated with markers of neutrophilic airway inflammation and absolute neutrophil counts. This supports the hypothesis that viable, non-apoptotic airway neutrophils contribute to CF lung disease (38, 39) and proposes that surface-bound NE activity rather depicts neutrophil activation at the single cell level than unspecific NE liberation from disintegrating cells.

In the lungs, α1-antitrypsin and SLPI are the most abundant serine antiproteases that counterbalance the harmful effects of excessive NE activity (34). The acute phase protein α1-antitrypsin is primarily produced in the liver, distributed via the blood stream and inactivates NE by forming stable complexes (34). SLPI is secreted locally in the airways by various cell types, including neutrophils, macrophages and airway epithelial cells, and is cleaved and inactivated by free NE (10). Studies on human BAL neutrophils have shown that surface-bound NE on non-adherent neutrophils can be fully inhibited by α1-antitrypsin, leading to a permanent clearance of NE from the cell surface (45). In our CF cohort, however, the antiprotease shield was considerably impaired, as SLPI was decreased and did not correlate with NE activity. Interestingly, free but not surface-bound NE activity correlated with NE-α1-antitrypsin-compexes. Therefore, we speculate that the high levels of free NE consume a significant proportion of α1-antitrypsin in advanced CF lung disease. Surface-

bound NE activity might represent a pool of freshly secreted NE, not inhibited by the antiproteasis shield.

Finally, we could show that NE activity on sputum neutrophils was associated with impaired lung function in adult patients with CF. Both, free and surface-bound NE activities correlated with FEV_1 % predicted, the most widely accepted pulmonary function test for disease progression in CF. Moreover, the novel FRET approach demonstrated that surface-bound, but not free NE activity, correlates with air trapping, a surrogate measure for airflow obstruction and structural lung damage in CF lung disease. This is in line with preclinical data in βENaC-overexpressing mice that identified surface-bound NE activity as a potent trigger of emphysematous airspace enlargement in CF-like lung disease (4). Recent CT studies have further highlighted the important pathophysiologic role of early-onset emphysema in patients with CF (46, 47). Mechanistically, It has been proposed that adherence of neutrophils to the site of proteolysis might protect surface-bound NE activity against endogenous antiproteases and thus aggravate local tissue damage (45). Taken together, these data suggest that surface-bound NE activity is associated with severity of CF lung disease and might be a sensitive measure for tissue destruction in CF airways.

NE inhibitors are proposed as a promising anti-inflammatory and tissue-protective therapy in CF lung disease (48–50). This study confirms that NE needs to be inhibited and monitored on the neutrophil membrane for optimal therapeutic efficiency (4). Recent clinical studies in 3-month-old infants with CF have shown that free NE activity in BAL fluid is the major risk factor for bronchiectasis at 12 month, but free NE activity was below the detection limit in more than 70 % of the analyzed samples (1, 2). This is similar to findings in βENaC-overexpressing mice, showing that free NE activity cannot be measured in cell-free BAL supernatans from mice with CF-like lung disease in presence of an intact antiprotease shield (4). Hence, more sensitive methods for the measurement of NE activity in airway secretions are required. The current study showed that even in adults with CF, surface-bound NE

activity can be increased when free NE activity is low. Further, our data have shown that surface-bound NE activity was independent from established markers of neutrophilic inflammation. This proposes that quantification of surface-bound NE activity could serve as a high-sensitivity approach for detection of neutrophilic inflammation in airway secretions.

An important limitation of this study is the single-center, cross-sectional design. Therefore, future longitudinal clinical studies are required to estimate the predictive value of surface-bound NE activity in stable CF lung disease and in exacerbation.

In summary, this study demonstrated for the first time that NE activity on sputum neutrophils is associated with severity of CF lung disease. Our data suggest that surface-bound NE activity might represent neutrophil activation rather than neutrophil number. Further, the antiprotease shield predominantly counteracts free NE activity while freshly secreted NE on the neutrophil surface might be protected from endogenous inhibitors. Correlations with lung function data revealed that surface-bound NE activity is associated with both airflow limitation and air trapping. Taken together, our results suggest that NE activity on airway neutrophils may play an important role in pathogenesis and could provide a valuable biomarker for monitoring of progression in CF lung disease.

ACKNOWLEDGEMENTS

The authors thank all patients with CF for their participation in this study, Y. Koch and S. Korsten for assistance in sample collection, and N. Heath, F. Stein, A. Halavatyi and M. Lampe (TLRC Microscopy Laboratory) for expert assistance in microscopy and data analysis.

REFERENCES

1. Sly PD, Brennan S, Gangell C, de Klerk N, Murray C, Mott L, Stick SM, Robinson PJ, Robertson CF, Ranganathan SC, Australian Respiratory Early Surveillance Team for Cystic Fibrosis (AREST-CF). Lung disease at diagnosis in infants with cystic fibrosis detected by newborn screening. *Am J Respir Crit Care Med* 2009;180:146–152.
2. Sly PD, Gangell CL, Chen L, Ware RS, Ranganathan S, Mott LS, Murray CP, Stick SM. Risk factors for bronchiectasis in children with cystic fibrosis. N Engl J Med 2013;368:1963–1970.
3. Sagel SD, Wagner BD, Anthony MM, Emmett P, Zemanick ET. Sputum biomarkers of inflammation and lung function decline in children with cystic fibrosis. Am J Respir Crit Care Med 2012;186:857–865.
4. Gehrig S, Duerr J, Weitnauer M, Wagner CJ, Graeber SY, Schatterny J, Hirtz S, Belaaouaj A, Dalpke AH, Schultz C, Mall MA. Lack of neutrophil elastase reduces inflammation, mucus hypersecretion, and emphysema, but not mucus obstruction, in mice with cystic fibrosis–like lung disease. Am J Respir Crit Care Med 2014;189:1082–1092.
5. Voynow JA, Fischer BM, Malarkey DE, Burch LH, Wong T, Longphre M, Ho SB, Foster WM. Neutrophil elastase induces mucus cell metaplasia in mouse lung. *Am J Physiol Lung Cell Mol Physiol* 2004;287:L1293-1302.
6. Fahy JV, Dickey BF. Airway Mucus Function and Dysfunction. *N Engl J Med* 2010;363:2233–2247.
7. Owen CA. Roles for proteinases in the pathogenesis of chronic obstructive pulmonary disease. *Int J Chron Obstruct Pulmon Dis* 2008;3:253–268.
8. Pham CTN. Neutrophil serine proteases: specific regulators of inflammation. *Nat Rev Immunol* 2006;6:541–550.
9. Lee WL, Downey GP. Leukocyte elastase: physiological functions and role in acute lung injury. *Am J Respir Crit Care Med* 2001;164:896–904.
10. Weldon S, McNally P, McElvaney NG, Elborn JS, McAuley DF, Wartelle J, Belaaouaj A, Levine RL, Taggart CC. Decreased levels of secretory leucoprotease inhibitor in the

Pseudomonas-infected cystic fibrosis lung are due to neutrophil elastase degradation. *J Immunol Baltim Md 1950* 2009;183:8148–8156.

11. Alexis NE, Muhlebach MS, Peden DB, Noah TL. Attenuation of host defense function of lung phagocytes in young cystic fibrosis patients. *J Cyst Fibros Off J Eur Cyst Fibros Soc* 2006;5:17–25.
12. Gibson RL, Burns JL, Ramsey BW. Pathophysiology and management of pulmonary infections in cystic fibrosis. *Am J Respir Crit Care Med* 2003;168:918–951.
13. Taggart CC, Greene CM, Carroll TP, O'Neill SJ, McElvaney NG. Elastolytic proteases: inflammation resolution and dysregulation in chronic infective lung disease. *Am J Respir Crit Care Med* 2005;171:1070–1076.
14. Roghanian A, Drost EM, MacNee W, Howie SEM, Sallenave J-M. Inflammatory lung secretions inhibit dendritic cell maturation and function via neutrophil elastase. *Am J Respir Crit Care Med* 2006;174:1189–1198.
15. Le Gars M, Descamps D, Roussel D, Saussereau E, Guillot L, Ruffin M, Tabary O, Hong S-S, Boulanger P, Paulais M, Malleret L, Belaaouaj A, Edelman A, Huerre M, Chignard M, Sallenave J-M. Neutrophil elastase degrades cystic fibrosis transmembrane conductance regulator via calpains and disables channel function in vitro and in vivo. *Am J Respir Crit Care Med* 2013;187:170–179.
16. Caldwell RA, Boucher RC, Stutts MJ. Neutrophil elastase activates near-silent epithelial Na+ channels and increases airway epithelial Na+ transport. *Am J Physiol Lung Cell Mol Physiol* 2005;288:L813-819.
17. Mall MA. Role of the amiloride-sensitive epithelial Na+ channel in the pathogenesis and as a therapeutic target for cystic fibrosis lung disease. *Exp Physiol* 2009;94:171–174.
18. Pillarisetti N, Williamson E, Linnane B, Skoric B, Robertson CF, Robinson P, Massie J, Hall GL, Sly P, Stick S, Ranganathan S, Australian Respiratory Early Surveillance Team for Cystic Fibrosis (AREST CF). Infection, inflammation, and lung function decline in infants with cystic fibrosis. *Am J Respir Crit Care Med* 2011;184:75–81.

19. Sagel SD, Sontag MK, Wagener JS, Kapsner RK, Osberg I, Accurso FJ. Induced sputum inflammatory measures correlate with lung function in children with cystic fibrosis. *J Pediatr* 2002;141:811–817.
20. Ratjen F, Waters V, Klingel M, McDonald N, Dell S, Leahy TR, Yau Y, Grasemann H. Changes in airway inflammation during pulmonary exacerbations in patients with cystic fibrosis and primary ciliary dyskinesia. *Eur Respir J* 2016;47:829–836.
21. Sagel SD, Kapsner R, Osberg I, Sontag MK, Accurso FJ. Airway inflammation in children with cystic fibrosis and healthy children assessed by sputum induction. *Am J Respir Crit Care Med* 2001;164:1425–1431.
22. Mayer-Hamblett N, Aitken ML, Accurso FJ, Kronmal RA, Konstan MW, Burns JL, Sagel SD, Ramsey BW. Association between pulmonary function and sputum biomarkers in cystic fibrosis. Am J Respir Crit Care Med 2007;175:822–828.
23. Owen CA. Leukocyte cell surface proteinases: regulation of expression, functions, and mechanisms of surface localization. *Int J Biochem Cell Biol* 2008;40:1246–1272.
24. Owen CA, Campbell MA, Boukedes SS, Campbell EJ. Cytokines regulate membrane-bound leukocyte elastase on neutrophils: a novel mechanism for effector activity. *Am J Physiol* 1997;272:L385-393.
25. Campbell EJ, Owen CA. The sulfate groups of chondroitin sulfate- and heparan sulfate-containing proteoglycans in neutrophil plasma membranes are novel binding sites for human leukocyte elastase and cathepsin G. *J Biol Chem* 2007;282:14645–14654.
26. Gehrig S, Mall MA, Schultz C. Spatially Resolved Monitoring of Neutrophil Elastase Activity with Ratiometric Fluorescent Reporters. *Angew Chem Int Ed* 2012;51:6258–6261.
27. Dittrich AS, Heath N, Wiebel M, Herth FJ, Schultz C, Mall MA. Neutrophil elastase activity on the surface of sputum neutrophils is associated with severity of cystic fibrosis lung disease [abstract]. Am J Respir Crit Care Med 2015;191:A6075.
28. Dittrich AS, Kühbandner I, Gehrig S, Rickert-Zacharias V, Taggart CC, Twigg M, Herth F, Schultz C, Mall MA. WS02.2 Role of soluble and membrane-associated neutrophil elastase activity in cystic fibrosis sputum [abstract]. J Cyst Fibros 2017;16:S3.

29. De Boeck K, Derichs N, Fajac I, de Jonge HR, Bronsveld I, Sermet I, Vermeulen F, Sheppard DN, Cuppens H, Hug M, Melotti P, Middleton PG, Wilschanski M, ECFS Diagnostic Network Working Group, EuroCareCF WP3 Group on CF diagnosis. New clinical diagnostic procedures for cystic fibrosis in Europe. *J Cyst Fibros Off J Eur Cyst Fibros Soc* 2011;10 Suppl 2:S53-66.
30. Farrell PM, White TB, Ren CL, Hempstead SE, Accurso F, Derichs N, Howenstine M, McColley SA, Rock M, Rosenfeld M, Sermet-Gaudelus I, Southern KW, Marshall BC, Sosnay PR. Diagnosis of cystic fibrosis: consensus guidelines from the cystic fibrosis foundation. *J Pediatr* 2017;181:S4–S15.e1.
31. Miller MR. Standardisation of spirometry. *Eur Respir J* 2005;26:319–338.
32. Wanger J, Clausen JL, Coates A, Pedersen OF, Brusasco V, Burgos F, Casaburi R, Crapo R, Enright P, van der Grinten CPM, Gustafsson P, Hankinson J, Jensen R, Johnson D, MacIntyre N, McKay R, Miller MR, Navajas D, Pellegrino R, Viegi G. Standardisation of the measurement of lung volumes. *Eur Respir J* 2005;26:511–522.
33. Hector A, Jonas F, Kappler M, Feilcke M, Hartl D, Griese M. Novel method to process cystic fibrosis sputum for determination of oxidative state. *Respiration* 2010;80:393–400.
34. Greene CM, McElvaney NG. Proteases and antiproteases in chronic neutrophilic lung disease - relevance to drug discovery: Proteases and antiproteases in lung disease. *Br J Pharmacol* 2009;158:1048–1058.
35. McGarvey LPA, Dunbar K, Martin SL, Brown V, Macmahon J, Ennis M, Elborn JS. Cytokine concentrations and neutrophil elastase activity in bronchoalveolar lavage and induced sputum from patients with cystic fibrosis, mild asthma and healthy volunteers. *J Cyst Fibros Off J Eur Cyst Fibros Soc* 2002;1:269–275.
36. Downey DG, Martin SL, Dempster M, Moore JE, Keogan MT, Starcher B, Edgar J, Bilton D, Elborn JS. The relationship of clinical and inflammatory markers to outcome in stable patients with cystic fibrosis. *Pediatr Pulmonol* 2007;42:216–220.
37. Nichols DP, Chmiel JF. Inflammation and its genesis in cystic fibrosis. *Pediatr Pulmonol* 2015;50 Suppl 40:S39-56.

38. Tirouvanziam R, Gernez Y, Conrad CK, Moss RB, Schrijver I, Dunn CE, Davies ZA, Herzenberg LA, Herzenberg LA. Profound functional and signaling changes in viable inflammatory neutrophils homing to cystic fibrosis airways. *Proc Natl Acad Sci* 2008;105:4335–4339.
39. Margaroli C, Tirouvanziam R. Neutrophil plasticity enables the development of pathological microenvironments: implications for cystic fibrosis airway disease. *Mol Cell Pediatr* 2016;3:.
40. Owen CA, Campbell MA, Sannes PL, Boukedes SS, Campbell EJ. Cell surface-bound elastase and cathepsin G on human neutrophils: a novel, non-oxidative mechanism by which neutrophils focus and preserve catalytic activity of serine proteinases. *J Cell Biol* 1995;131:775–789.
41. Mall M, Grubb BR, Harkema JR, O'Neal WK, Boucher RC. Increased airway epithelial Na+ absorption produces cystic fibrosis-like lung disease in mice. *Nat Med* 2004;10:487–493.
42. Mall MA, Graeber SY, Stahl M, Zhou-Suckow Z. Early cystic fibrosis lung disease: Role of airway surface dehydration and lessons from preventive rehydration therapies in mice. *Int J Biochem Cell Biol* 2014;52:174–179.
43. Mall MA, Harkema JR, Trojanek JB, Treis D, Livraghi A, Schubert S, Zhou Z, Kreda SM, Tilley SL, Hudson EJ, O'Neal WK, Boucher RC. Development of chronic bronchitis and emphysema in β-epithelial Na + channel–overexpressing mice. *Am J Respir Crit Care Med* 2008;177:730–742.
44. Zhou Z, Duerr J, Johannesson B, Schubert SC, Treis D, Harm M, Graeber SY, Dalpke A, Schultz C, Mall MA. The ENaC-overexpressing mouse as a model of cystic fibrosis lung disease. *J Cyst Fibros Off J Eur Cyst Fibros Soc* 2011;10 Suppl 2:S172-182.
45. Korkmaz B, Attucci S, Jourdan M-L, Juliano L, Gauthier F. Inhibition of neutrophil elastase by 1-protease inhibitor at the surface of Human polymorphonuclear neutrophils. *J Immunol* 2005;175:3329–3338.
46. Wielpütz MO, Weinheimer O, Eichinger M, Wiebel M, Biederer J, Kauczor H-U, Heußel CP, Mall MA, Puderbach M. Pulmonary emphysema in cystic fibrosis detected by densitometry on chest multidetector computed tomography. PLoS ONE 2013;8:e73142.

47. Mets OM, Roothaan SM, Bronsveld I, Luijk B, van de Graaf EA, Vink A, de Jong PA. Emphysema is common in lungs of cystic fibrosis lung transplantation patients: a histopathological and computed tomography study. PLOS ONE 2015;10:e0128062.
48. Gaggar A, Chen J, Chmiel JF, Dorkin HL, Flume PA, Griffin R, Nichols D, Donaldson SH. Inhaled alpha1-proteinase inhibitor therapy in patients with cystic fibrosis. *J Cyst Fibros Off J Eur Cyst Fibros Soc* 2016;15:227–233.
49. Chillappagari S, Müller C, Mahavadi P, Guenther A, Nährlich L, Rosenblum J, Rubin BK, Henke MO. A small molecule neutrophil elastase inhibitor, KRP-109, inhibits cystic fibrosis mucin degradation. *J Cyst Fibros Off J Eur Cyst Fibros Soc* 2016;15:325–331.
50. Griese M, Latzin P, Kappler M, Weckerle K, Heinzlmaier T, Bernhardt T, Hartl D. alpha1-Antitrypsin inhalation reduces airway inflammation in cystic fibrosis patients. *Eur Respir J* 2007;29:240–250.

FIGURE LEGENDS

Figure 1. NE activity is increased on the surface of CF sputum neutrophils. (A-B) Absolute (A) and relative (B) number of neutrophils in control and CF sputum. (C) Representative ratio images calculated from donor and acceptor fluorescence of sputum neutrophils from a healthy non-smoker (control) and a patient with cystic fibrosis (CF). (D-E) Free NE activity in sputum supernatants (D) and cell surface-bound NE activity on sputum neutrophils (E) from controls and patients with CF in the absence and presence of the NE inhibitor sivelestat. (F) Correlation between surface-bound and free NE activity in CF sputum. (G) Surface-bound NE activity on neutrophils from controls (Con) and stratified according to quartile groups of free NE activity in sputum from patients with CF (25: ≤25th percentile, 50: >25th-50th percentile, 75: >50th-75th percentile and 100: >75th-100th percentile). Dots represent individual samples and lines represent the group median. *P<0.05, **P<0.01 and ***P<0.001 compared with control. $^{\dagger}P$<0.001 compared with CF without sivelestat

Figure 2. Relationship of free and surface-bound NE activity with parameters of neutrophilic inflammation and antiproteases in CF sputum. (A-J) Correlations of free NE activity in sputum supernatant (A,C,E,G,I) and surface-bound NE activity on sputum neutrophils (B,D,F,H,J) with neutrophil counts (A and B), and levels of interleukin-8 (IL-8) (C and D), myeloperoxidase (MPO) (E and F), α1-antitypsin-NE complexes (AAT-NE) (G and H) and secretory leukocyte protease inhibitor (SLPI) (I and J) in CF sputum. Spearman correlation coefficient r and P values are provided for each correlation.

Figure 3. Relationship of free and surface-bound NE activity with lung function parameters of airflow obstruction and air trapping in patients with CF. (A-D) Correlations of free NE activity in sputum supernatant and surface-bound NE activity on sputum neutrophils with forced expiratory volume in one second % predicted (FEV_1 % pred.) (A and B) and plethysmographic functional

residual capacity % predicted (FRCpleth % pred.) (C and D). Spearman correlation coefficient r and *P* values are provided for each correlation.

TABLES

Table 1. Clinical characteristics of patients with cystic fibrosis

Number of subjects	n	35
Number of visits	n	39
Age (years)	Median (IQR)	27.7 (23.8–30.8)
	Range	19.1 – 59.0
Sex	n, males/females	23/12
^{2}	Median (IQR)	19.79 (18.88–21.43)
	Range	16.35– 27.72
FEV % predicted	Median (IQR)	53.30 (38.70–68.10)
	Range	18.00 – 114.80
FRCpleth % predicted*	Median (IQR)	140.40 (113.78–169.38)
	Range	89.60–212.40
CFTR genotype		
F508del/F508del	n (Percentage)	13/35 (37.14%)
F508del/other	n (Percentage)	19/35 (54.29%)
other/other	n (Percentage)	3/35 (8.57%)
Pseudomonas infection		
negative	n (Percentage)	13/39 (33.33%)
intermittent	n (Percentage)	3/39 (7.69%)
chronic	n (Percentage)	23/39 (58.97%)
Pancreatic insufficiency	n (Percentage)	30/35 (83.33%)

Definition of abbreviations: BMI: Body mass index, FEV_1 % predicted: forced expiratory volume in one second % predicted, FRCpleth % predicted: plethysmographic functional residual capacity % predicted, IQR: interquartile range 25–75th percentile*FRCpleth % predicted determined by body plethysmography was available in 28 of 39 visits.

Table 2.Myeloperoxidase, antiproteases and IL-8 in control and CF sputum

	Control	CF	*P*-value
MPO	0.41 (0.32–0.55)	29.69 (18.44–51.81)	<0.001
SLPI (ng/mL)	1211.55 (923.27–1811.72)	180.81 (87.30–474.10)	<0.001
AAT-NE (ng/mL)	24.84 (15.62–33.24)	82.57 (40.41–183.14)	<0.001
IL-8 (ng/mL)	0.56 (0.29–0.80)	11.15 (8.23–16.58)	<0.001

Definition of abbreviations: MPO: myeloperoxidase, SLPI: secretory leukocyte protease inhibitor, AAT-NE: α1-antitrypsin-NE complexes, IL-8: interleukin-8.

FIGURES

Figure 1

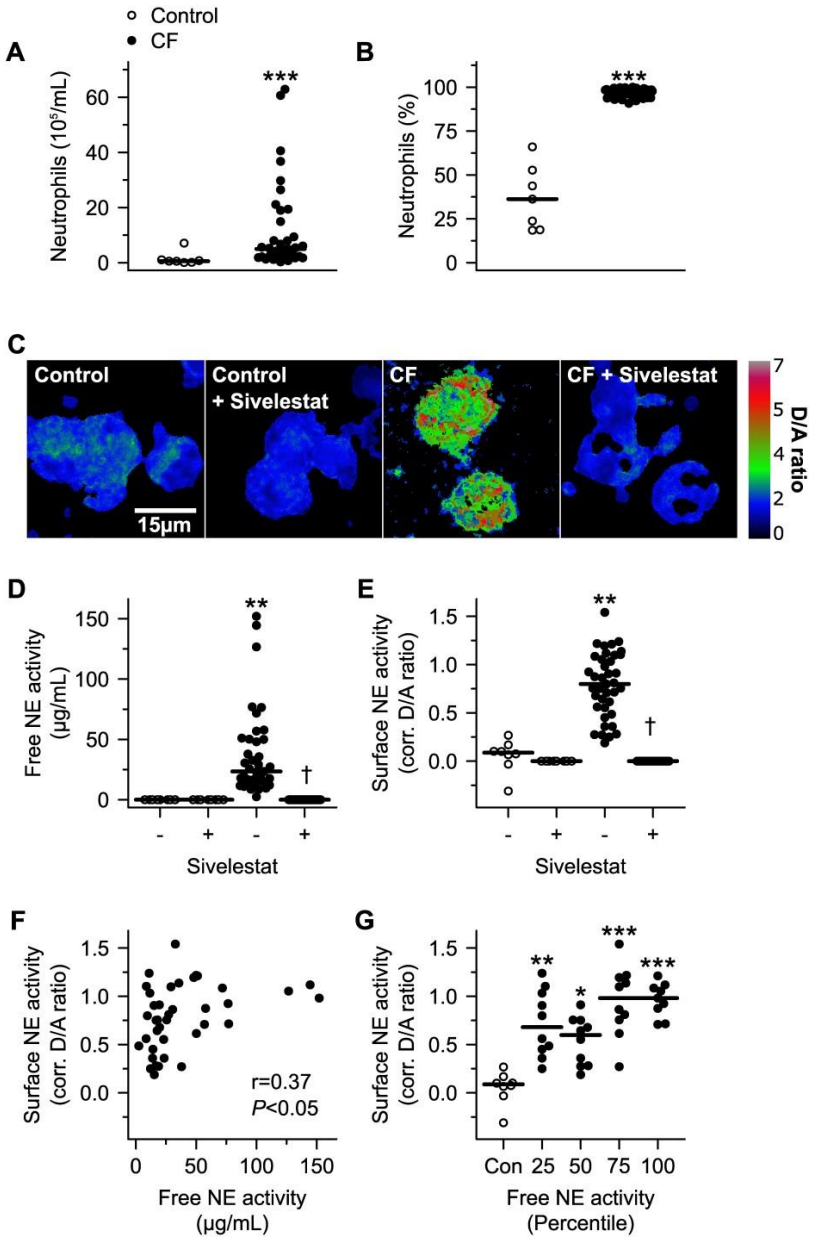


Figure 2

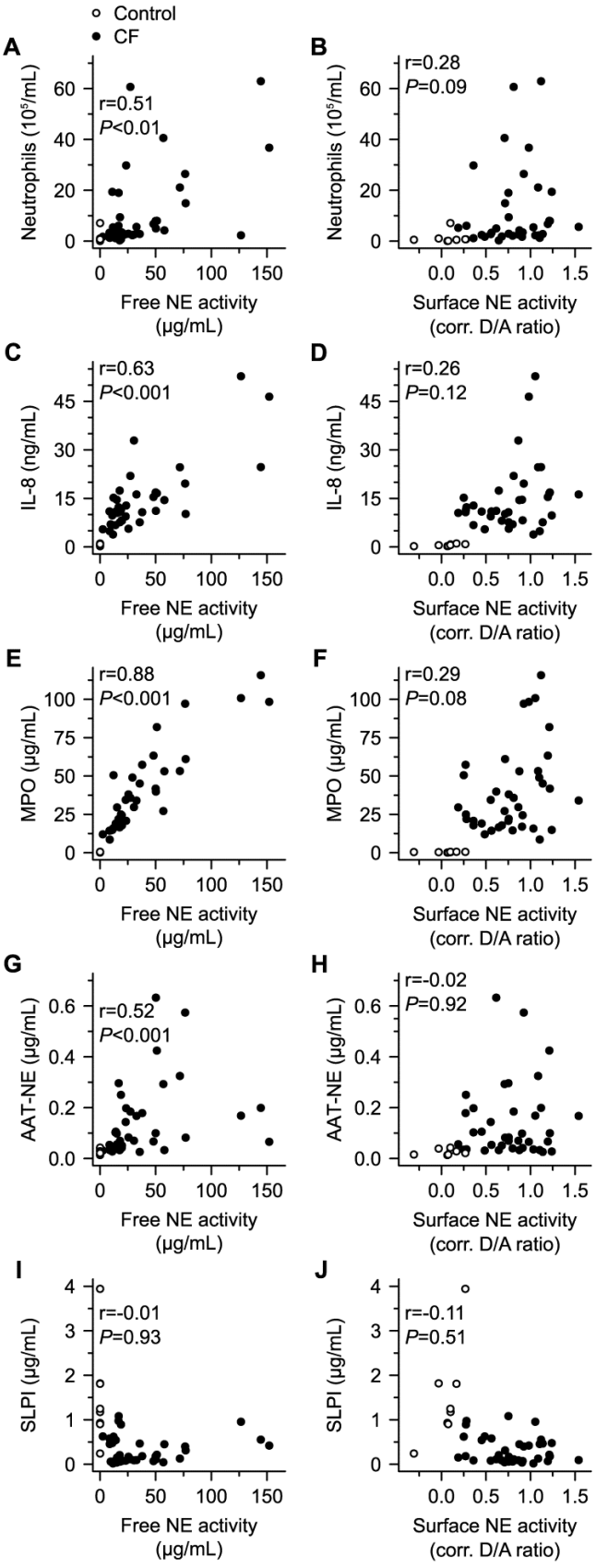
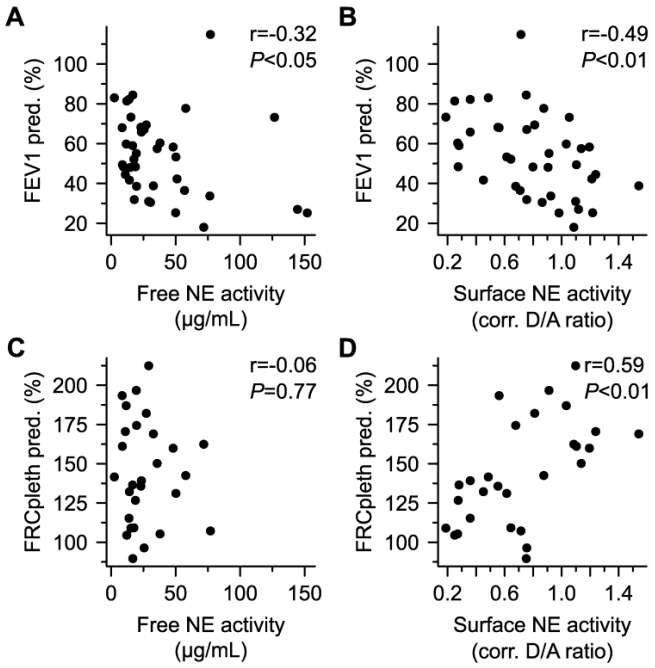

Control
CF
A
Neutrophils (10⁵/mL)
r=0.51
P<0.01
Free NE activity (µg/mL)
B
r=0.28
P=0.09
Surface NE activity (corr. D/A ratio)
C
IL-8 (ng/mL)
r=0.63
P<0.001
D
r=0.26
P=0.12
E
MPO (µg/mL)
r=0.88
P<0.001
F
r=0.29
P=0.08
G
AAT-NE (µg/mL)
r=0.52
P<0.001
H
r=-0.02
P=0.92
I
SLPI (µg/mL)
r=-0.01
P=0.93
J
r=-0.11
P=0.51

Figure 3


A
r=-0.32
P<0.05
FEV1 pred. (%)
100
80
60
40
20
0
50
100
150
Free NE activity
(µg/mL)
B
r=-0.49
P<0.01
FEV1 pred. (%)
100
80
60
40
20
0.2
0.6
1.0
1.4
Surface NE activity
(corr. D/A ratio)
C
r=-0.06
P=0.77
FRCpleth pred. (%)
200
175
150
125
100
0
50
100
150
Free NE activity
(µg/mL)
D
r=0.59
P<0.01
FRCpleth pred. (%)
200
175
150
125
100
0.2
0.6
1.0
1.4
Surface NE activity
(corr. D/A ratio)