



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

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1 **Elastase activity on sputum neutrophils correlates with severity of** 2 **lung disease in cystic fibrosis**

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46 **AT A GLANCE COMMENTARY**

47 **Scientific knowledge on the subject**

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

54

55 **What this study adds to the field**

56 This study investigated the relationship between NE activity on the surface of sputum
57 neutrophils and severity of lung disease in adult patients with CF. We demonstrate that NE
58 activity is increased on the surface of CF sputum neutrophils, even at low levels of free NE
59 activity in cell-free sputum supernatants. Further, cell surface-bound NE activity correlated
60 inversely with FEV₁ % predicted and FRCpleth % predicted as outcome measures of airflow
61 limitation and air trapping. These results suggest that surface-bound NE activity may
62 contribute to severity of lung disease and serve as a novel biomarker in patients with CF.

63

64

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

67

68

69 **ABSTRACT**

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

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

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

81 **Measurement and main results** Surface-bound NE activity on sputum neutrophils was
82 increased in CF compared to healthy controls ($P<0.01$) and correlated with free NE activity
83 ($P<0.05$), but not with other inflammation markers in CF sputum. Surface-bound and free NE
84 activity correlated with FEV₁ % predicted ($P<0.01$ and $P<0.05$), but only surface-bound NE
85 activity correlated with FRCpleth % predicted ($P<0.01$).

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

91

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93

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

96 **INTRODUCTION**

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

119

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

124 bound NE activity on sputum neutrophils and free NE activity in sputum supernatants.
125 Further, we determined sputum neutrophil counts and levels of interleukin-8 (IL-8),
126 myeloperoxidase (MPO) and antiproteases such as α 1-antitrypsin-NE complexes (AAT-NE)
127 and secretory leukocyte protease inhibitor (SLPI) in sputum supernatant, performed paired
128 pulmonary function testing, and correlated surface-bound NE activity with these markers of
129 disease severity in patients with CF. Some of the results have been previously reported in
130 the form of abstracts (27, 28).

131

132

133 **METHODS**

134 **Study population**

135 This study was approved by the ethics committee of the University of Heidelberg and
136 informed written consent was obtained from all subjects. The diagnosis of CF was verified by
137 established diagnostic criteria (29, 30). Spontaneously expectorated (n=36) or induced
138 sputum (n=3) of sufficient quantity (total cell count of inflammatory cells >60,000) was
139 collected from 35 clinically stable patients with CF during routine visits at the CF Center at
140 the University Hospital Heidelberg. Patient characteristics are summarized in Table 1 and
141 *CFTR* genotypes are provided in Table E1. Colonization with *Pseudomonas aeruginosa* was
142 defined as negative when 0%, intermittent when \leq 50% and chronic when >50% of airway
143 cultures were positive in the previous twelve month. Airflow obstruction and pulmonary
144 hyperinflation were detected by measurement of forced expiratory volume in one second
145 (FEV₁) and plethysmographic functional residual capacity (FRC_{pleth}) on the same day of
146 sputum collection according to ATS/ERS guidelines (31, 32). As control group, we collected
147 induced sputum from 8 age-matched, healthy, non-smoking volunteers (5 females and 3
148 males; median age 31.1 range 22.01-45.02 years) by hypertonic saline inhalation as
149 previously described (4, 33). Briefly, the mouth was rinsed with water and 100 μ g salbutamol
150 was inhaled prior to 6% hypertonic saline. Sputum samples were directly collected in a
151 specimen container, stored on ice immediately after production and processed within two

152 hours.

153

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

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

168

169 **Statistical analysis**

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

178

179

180 **RESULTS**

181 **NE activity is increased on the surface of sputum neutrophils in CF, even in patients** 182 **with low levels of free NE activity in sputum supernatant**

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

203

204 **Relationship of surface-bound and free NE activity with markers of neutrophilic** 205 **inflammation and antiproteases**

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

208 antiproteases that inhibit NE activity in the airway lumen. Free NE activity in CF sputum
209 supernatants correlated with absolute neutrophil counts, IL-8 and MPO (Figures 2A, C and
210 E). Surface-bound NE activity did not correlate with these markers of neutrophilic
211 inflammation (Figures 2 B, D and F). Neither free nor surface-bound NE activity correlated
212 with percentage of sputum neutrophils ($r=0.11$, $P=0.53$ and $r=0.15$, $P=0.36$). To investigate
213 the relationship of free and surface-bound NE activity with antiproteases, we measured
214 levels of SLPI and complexes of NE with α 1-antitrypsin (AAT-NE), two major endogenous
215 inhibitors of NE in the airways (34), in sputum supernatants. As expected from previous
216 studies (10, 35), AAT-NE was significantly increased ($P<0.001$), whereas SLPI was
217 decreased ($P<0.001$) in CF compared to control sputum (Table 2). Free, but not surface-
218 bound NE activity correlated with AAT-NE (Figure 2G and H). Neither free, nor surface-
219 bound NE activity were correlated with SLPI (Figure 2I and J). Collectively, these data
220 indicate that free NE activity depends on the level of airway inflammation, as determined
221 from the number of neutrophils, levels of IL-8 and AAT-NE in sputum, whereas surface-
222 bound NE activity is increased on CF neutrophils independent of these indices of neutrophilic
223 airway inflammation.

224

225 **Surface-bound NE activity on sputum neutrophils correlates with airflow obstruction** 226 **and air trapping**

227 To determine the clinical relevance of increased NE activity on the surface of CF neutrophils,
228 we correlated free and surface-bound NE activity with FEV₁ and (FRCpleth) as lung function
229 parameters of airflow obstruction and air trapping. As expected from previous studies (3, 19,
230 22, 36), free NE activity in CF sputum showed a negative correlation with FEV₁ % predicted
231 ($P<0.05$) in our cohort of patients with CF (Figure 3A), whereas no relationship was found
232 between free NE and FRCpleth % predicted (Figure 3C). Surface-bound NE activity on CF
233 sputum neutrophils was also inversely correlated with FEV₁ % predicted ($P<0.01$, Figure 3B).
234 In addition, surface-bound NE activity was directly correlated with FRC % predicted in
235 patients with CF ($P<0.01$, Figure 3D). These results show that the level of surface-bound NE

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

238

239

240 **DISCUSSION**

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

255

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

264 supernatants of BAL fluid from β ENaC-overexpressing mice, whereas surface-bound NE
265 activity on BAL neutrophils was constantly increased. This was likely due to an intact
266 antiprotease shield, as BAL supernatants could inhibit activity of purified NE in a dose-
267 dependent manner (4). So far, the massive influx, necrosis, cell lysis and impaired clearance
268 of neutrophils has been considered as the main mechanism leading to NE burden in
269 advanced CF lung disease (37). In our cohort, sputum neutrophil counts were markedly
270 elevated, consistent with reported neutrophil numbers in sputum from adult patients with CF
271 (4, 20). However, we found that surface-bound NE activity was increased on sputum
272 neutrophils, even when free NE activity was low. Further, free but not surface-bound NE
273 activity correlated with markers of neutrophilic airway inflammation and absolute neutrophil
274 counts. This supports the hypothesis that viable, non-apoptotic airway neutrophils contribute
275 to CF lung disease (38, 39) and proposes that surface-bound NE activity rather depicts
276 neutrophil activation at the single cell level than unspecific NE liberation from disintegrating
277 cells.

278

279 In the lungs, α 1-antitrypsin and SLPI are the most abundant serine antiproteases that
280 counterbalance the harmful effects of excessive NE activity (34). The acute phase protein
281 α 1-antitrypsin is primarily produced in the liver, distributed via the blood stream and
282 inactivates NE by forming stable complexes (34). SLPI is secreted locally in the airways by
283 various cell types, including neutrophils, macrophages and airway epithelial cells, and is
284 cleaved and inactivated by free NE (10). Studies on human BAL neutrophils have shown that
285 surface-bound NE on non-adherent neutrophils can be fully inhibited by α 1-antitrypsin,
286 leading to a permanent clearance of NE from the cell surface (45). In our CF cohort,
287 however, the antiprotease shield was considerably impaired, as SLPI was decreased and did
288 not correlate with NE activity. Interestingly, free but not surface-bound NE activity correlated
289 with NE- α 1-antitrypsin-complexes. Therefore, we speculate that the high levels of free NE
290 consume a significant proportion of α 1-antitrypsin in advanced CF lung disease. Surface-

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

293

294 Finally, we could show that NE activity on sputum neutrophils was associated with impaired
295 lung function in adult patients with CF. Both, free and surface-bound NE activities correlated
296 with FEV₁ % predicted, the most widely accepted pulmonary function test for disease
297 progression in CF. Moreover, the novel FRET approach demonstrated that surface-bound,
298 but not free NE activity, correlates with air trapping, a surrogate measure for airflow
299 obstruction and structural lung damage in CF lung disease. This is in line with preclinical data
300 in β ENaC-overexpressing mice that identified surface-bound NE activity as a potent trigger of
301 emphysematous airspace enlargement in CF-like lung disease (4). Recent CT studies have
302 further highlighted the important pathophysiologic role of early-onset emphysema in patients
303 with CF (46, 47). Mechanistically, It has been proposed that adherence of neutrophils to the
304 site of proteolysis might protect surface-bound NE activity against endogenous antiproteases
305 and thus aggravate local tissue damage (45). Taken together, these data suggest that
306 surface-bound NE activity is associated with severity of CF lung disease and might be a
307 sensitive measure for tissue destruction in CF airways.

308

309 NE inhibitors are proposed as a promising anti-inflammatory and tissue-protective therapy in
310 CF lung disease (48–50). This study confirms that NE needs to be inhibited and monitored
311 on the neutrophil membrane for optimal therapeutic efficiency (4). Recent clinical studies in
312 3-month-old infants with CF have shown that free NE activity in BAL fluid is the major risk
313 factor for bronchiectasis at 12 month, but free NE activity was below the detection limit in
314 more than 70 % of the analyzed samples (1, 2). This is similar to findings in β ENaC-
315 overexpressing mice, showing that free NE activity cannot be measured in cell-free BAL
316 supernatans from mice with CF-like lung disease in presence of an intact antiprotease shield
317 (4). Hence, more sensitive methods for the measurement of NE activity in airway secretions
318 are required. The current study showed that even in adults with CF, surface-bound NE

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

323

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

327

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

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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: $\leq 25^{\text{th}}$ percentile, 50: $>25^{\text{th}}-50^{\text{th}}$ percentile, 75: $>50^{\text{th}}-75^{\text{th}}$ percentile and 100: $>75^{\text{th}}-100^{\text{th}}$ percentile). Dots represent individual samples and lines represent the group median. * $P < 0.05$, ** $P < 0.01$ and *** $P < 0.001$ compared with control. † $P < 0.001$ 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), $\alpha 1$ -antitrypsin-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 (FEV₁ % 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 ₁ % 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, FEV₁ % 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	<i>P</i>-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: α 1-antitrypsin-NE complexes, IL-8: interleukin-8.

FIGURES

Figure 1

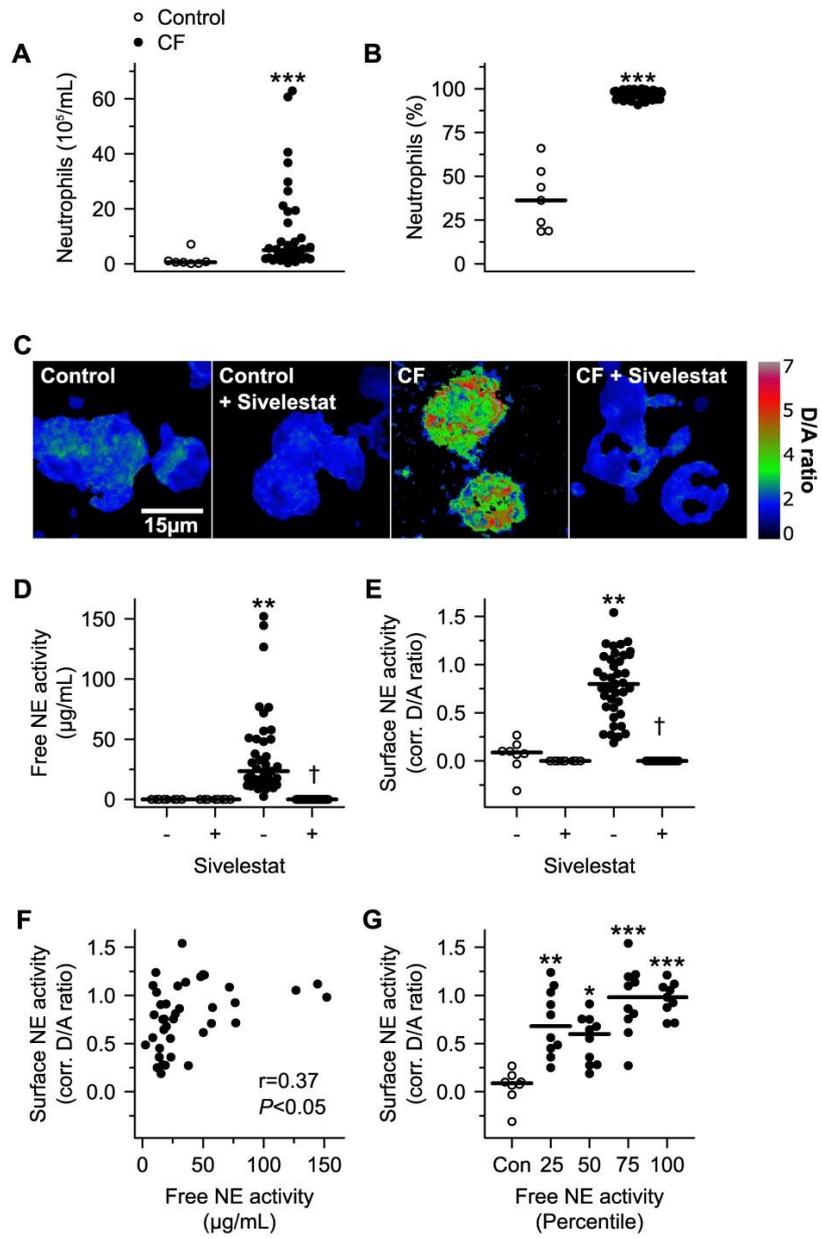


Figure 2

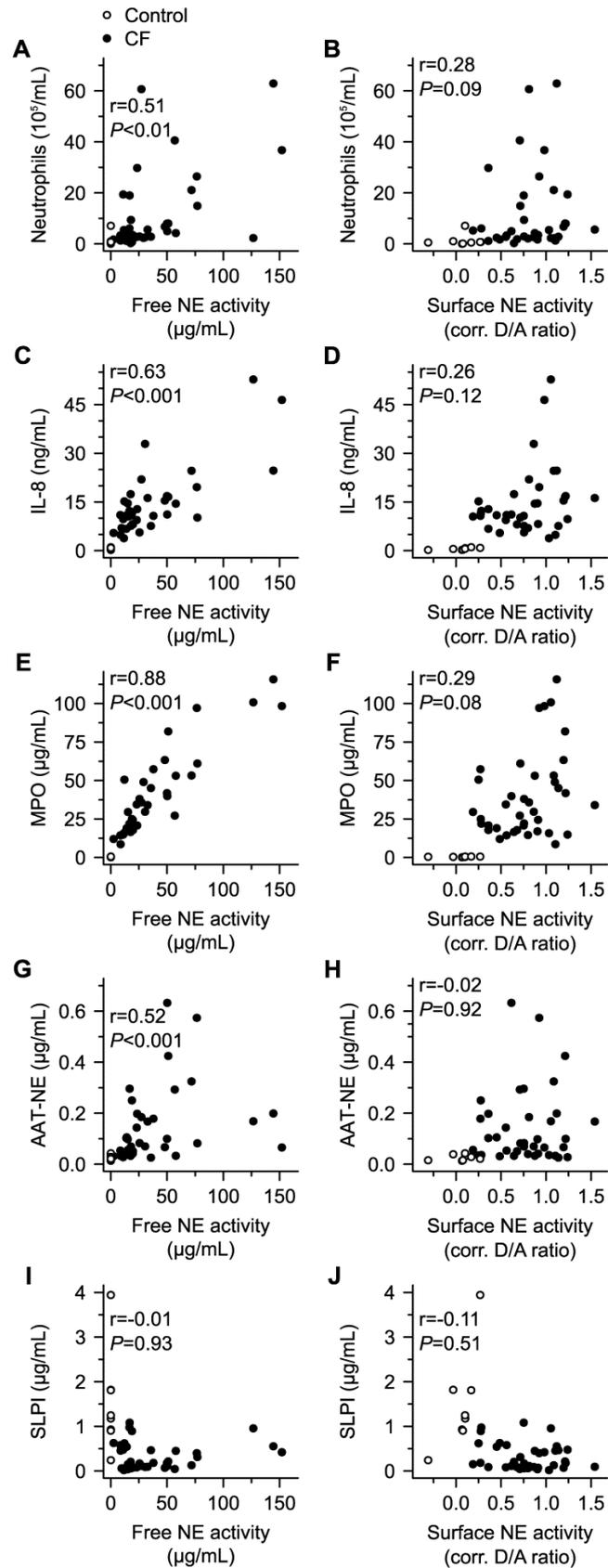


Figure 3

