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Neutrophil elastase activity is associated with exacerbations and lung function decline in bronchiectasis

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Running head: Neutrophil Elastase is a biomarker of disease progression and severity in bronchiectasis

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Scientific Knowledge on the Subject: There are no validated biomarkers of disease severity and progression in bronchiectasis. Studies in cystic fibrosis, and pilot studies in non-CF bronchiectasis suggest that neutrophil elastase is associated with more severe disease and airway bacterial infection. We prospectively tested the hypothesis that exacerbations and lung function decline is associated with increased sputum neutrophil elastase activity and the related circulating biomarker desmosine.

What this study adds to the field: Neutrophil elastase was associated with clinical and radiological extent of disease and with lung function. During follow-up, elevated levels of sputum neutrophil elastase activity identified patients at higher risk of exacerbations and severe exacerbations requiring hospital admission over 3 years. Sputum elastase activity was also independently associated with lung function decline. Increased circulating desmosine was also associated with a higher risk of severe exacerbations. As few clinical parameters have been shown to be associated with bronchiectasis outcomes, sputum neutrophil elastase and circulating desmosine may be useful adjuncts to clinical assessment, or to patient evaluation in clinical trials.

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

Abstract

Rationale: Sputum neutrophil elastase and serum desmosine, a linked marker of endogenous elastin degradation, are possible biomarkers of disease severity and progression in bronchiectasis. This study aimed to determine the association of elastase activity and desmosine with exacerbations and lung function decline in bronchiectasis.

Methods: This was a single-centre prospective cohort study using the TAYBRIDGE registry in Dundee, UK. 433 patients with HRCT-confirmed bronchiectasis provided blood samples for desmosine measurement and 381 provided sputum for baseline elastase activity measurements using an activity based immunosassay and fluorometric substrate assay. Candidate biomarkers were tested for their relationship with cross-sectional markers of disease severity, and with future exacerbations, mortality and lung function decline over 3-years.

Results: Elastase activity in sputum was associated with the bronchiectasis severity index ($r=0.49, p<0.0001$) and also correlated with MRC dyspnoea score ($r=0.34, p<0.0001$), FEV_1 % predicted ($r=-0.33, p<0.0001$) and the radiological extent of bronchiectasis ($r=0.29, p<0.0001$). During 3-years follow-up, elevated sputum elastase activity was associated with a higher frequency of exacerbations ($p<0.0001$) but was not independently associated with mortality. Sputum elastase activity was independently associated with FEV_1 decline (beta coefficient -0.139, $p=0.001$). Elastase showed good discrimination for severe exacerbations AUC 0.75 (0.72-0.79) and all-cause mortality AUC 0.70 (0.67-0.73) Sputum elastase activity increased at exacerbation ($p=0.001$) and was responsive to treatment with antibiotics.

Desmosine was correlated with sputum elastase ($r=0.34, p<0.0001$), and was associated with risk of severe exacerbations HR 2.7 (1.42-5.29), $p=0.003$, but not lung function decline.

Conclusions: Sputum neutrophil elastase activity is a biomarker of disease severity and future risk in adults with bronchiectasis.

Word count: 250

Indexing terms: bronchiectasis; neutrophils; inflammation; biomarker; exacerbations

Introduction

Bronchiectasis is characterised by permanent bronchial dilatation associated with chronic neutrophilic airway inflammation.(1) The pathogenesis of bronchiectasis is poorly understood but activated neutrophils are thought to be a key component of the "vicious cycle" of lung damage.(2) Neutrophil elastase (NE) is a 29-kDa serine protease stored in azurophilic granules which may be released during degranulation, neutrophil extracellular trap (NET) formation, or cell death.(3-8) NE is pro-inflammatory, slows ciliary beat frequency and stimulates mucus secretion.(9,10) It is found in high concentrations in the sputum of patients with neutrophilic lung diseases including bronchiectasis, COPD and cystic fibrosis(CF).(5-7) and it is thought that unopposed action of NE directly contributes to the pathogenesis and progression of these diseases.

NE activity is inhibited by antiproteases including secretory leukoproteinase inhibitor produced by bronchial epithelium and by serum derived α-1 antitrypsin.(11) In addition, the presence of high concentrations of DNA released during NET formation inhibits elastase activity, both directly and indirectly by modulating the response to NE inhibitors.(12) Epithelial derived factors such as the syndecan-1 also complex with elastase in the airway and reduce the inhibitory capacity of α-1 antitrypsin.(7,12)

Thus the activity of NE within the inflamed airway is usually controlled by a range of inhibitors. In bronchiectasis, however, release of NE overwhelms anti-proteinase defence leading to detectable levels of NE proteolytic activity in sputum and bronchoalveolar lavage.(13-15) This can be measured readily using assays detecting cleavage of chromogenic or fluorogenic peptide-based substrates in sputum, or downstream by measuring the endogenous degradation of mature elastin through the quantification of the unique covalent cross-linking amino acids desmosine and isodesmosine in serum/plasma (circulating desmosine-cDES).(16,17)

There are no widely accepted biomarkers of disease progression in bronchiectasis, but evidence is accumulating that sputum NE activity correlates with disease severity. Tsang *et al* showed in a study

of 30 patients that NE activity correlated strongly with 24h sputum volume, extent of bronchiectasis and forced expiratory volume in 1 second (FEV_1).(13) In 385 patients with bronchiectasis from the UK, NE activity correlated with airway bacterial load, the presence of *P. aeruginosa* and the extent of radiological bronchiectasis.(14) No previous study has investigated the association of sputum NE activity or cDES with clinically relevant outcomes in bronchiectasis during long term follow-up. In this study we prospectively tested the hypothesis that elevated sputum NE activity or the related biomarker cDES is associated with increased frequency of exacerbations and lung function decline.

Methods

This study was conducted and is reported according to Strengthening the Reporting of Observational Studies in Epidemiology (STROBE) guidelines.(18) Patients were consecutively recruited to a prospective observational study (TAYBRIDGE registry) at Ninewells Hospital, Dundee, UK 2012-2015. The study was approved by the East of Scotland Research Ethics committee (12/ES/0059) and all patients gave written informed consent. Inclusion criteria were age≥18 years, HRCT-confirmed bronchiectasis and clinical symptoms consistent with bronchiectasis. Exclusion criteria were; inability to give informed consent, active non-tuberculous mycobacterial infection, active allergic bronchopulmonary aspergillosis (ABPA), active tuberculosis, active malignancy, CF or pulmonary fibrosis with secondary traction bronchiectasis.

For inclusion in the present analysis, patients were asked to provide serum and sputum samples at the same baseline visit when clinically stable (defined as no antibiotic treatment within the preceding 4 weeks, excluding prophylactic oral or inhaled antibiotics).

Clinical assessement

Full details of the clinical assessments are shown in the online supplement. The underlying cause of bronchiectasis was determined by standardised testing according to British Thoracic Society (BTS) recommendations.(19) The Bronchiectasis Severity Index (BSI) was calculated as described.(20) Quality of life was evaluated using the St.Georges Respiratory Questionnaire (SGRQ).(21) Chronic infection was defined as the isolation of pathogens on at least 2 occasions 3 months apart during the preceding 12 months. (22) Spirometry was performed according to ATS/ERS guidelines. (23) The severity of radiological bronchiectasis was evaluated using the Reiff score. (24) Exacerbations were defined according to BTS recommendations and severe exacerbations were defined as those requiring hospital admission.(19)

Sputum sampling and processing

Spontaneous sputum samples were split for microbiology and inflammatory marker measurement. For measurement of inflammatory markers, including NE, spontaneous sputum was ultracentrifuged at 50,000g for 90 mins and the soluble fraction carefully removed. (14)

Methods of measurement of neutrophil elastase activity and other inflammatory markers

As previous studies have used several different methods of NE quantification, we simultaneously evaluated 3 methods in this study; two assays for sputum NE activity and one for cDES measurement.

Active NE was measured using an Activity Based Immunoassay (ProAxsis Ltd) [ABI, ProteaseTag® Active NE Immunoassay referred to as the ABI-NE assay] in accordance with the manufacturer's instructions (25,26) and a fluorogenic substrate-based kinetic assay (referred to as Kinetic-NE assay). Kinetic-NE employed the substrate N-Methoxysuccinyl-Ala-Ala-Pro-Val-7-amido-4-methylcoumarin (Sigma-Aldrich).

Sputum samples were assayed at dilutions ranging from 5x to 2000x and assays remaining below the lowest limit of detection (0.016μg/ml) at 5x dilution were recorded as 0 for the purposes of analysis.

Measurement of serum and sputum inflammatory markers (CXCL8, interleukin-1β, Tumour necrosis factor alpha (TNF-α) and EN-RAGE) were performed using commercially available ELISA. Prior to use, kits were validated for use in sputum according to the methods described by Woolhouse et al. (27)

Serum desmosine measurement

cDES was measured in serum using a validated LC-MS/MS method as previously described. (16, 17)

Exacerbation study

Patients (n=26), included in the main study, attending hospital for a severe exacerbation of bronchiectasis were enrolled in a substudy of changes in NE during exacerbations.(19) Spontaneous sputum samples were collected on day 1 prior to commencement of antibiotics and following treatment at day 14. Patients received standardised treatment for 14-days based on their previous sputum microbiology.(19) These 26 patients subsequently had additional sampling 6-months post-exacerbation to determine dynamics of NE.

Statistical analysis

Mean and standard deviation were used to display continuous normally distributed data with median and interquartile range for continuous non-normally distributed data, and frequencies and percentages for categorical data. The association of biomarkers with linear variables was performed using Spearman correlation, while between group differences were evaluated by ANOVA or Kruskal-Walis test. Frequency of exacerbations and severe exacerbations were evaluated using Poisson regression adjusted for duration of follow-up. Time to event data (time to first exacerbation, first hospital admission and death) were analysed using Kaplan-Meier survival analysis and Cox proportional hazard regression for multivariable analyses. Discrimination for mortality and severe exacerbations at 3-years was analysed using the area under a receiver operator characteristic curve (AUC). Analysis of FEV1 decline over 3 years was performed using multiple linear regression with appropriateness of the linear regression modelling evaluated by examining the distribution of residuals. In some analyses, patients were split into 3 groups based on low, intermediate and high elastase levels, with cut-offs selected using Youden's index. Patients with missing data were

excluded from analysis of the specific test as outlined below. No imputation methods were used. Sample size was empirical based on previous studies with equivalent lengths of follow-up. (14)

Results

Patient cohort

The study included 433 patients of whom 381 patients were able to provide a sputum sample sufficient for measurement of NE activity. The flow of patients through the study are shown in the STROBE flowchart (Figure 1)

Characteristics of the included patients are shown in Table 1. The median (IQR) age was 67 years (58-74); 60.7% of patients were female and 45% of patients had idiopathic bronchiectasis. The median exacerbation frequency was 1 per year (interquartile range 0-3). The median BSI score was 6 indicating a population with moderate-severe bronchiectasis (range 0-24).

There were no significant differences between patients able and unable to produce sputum. 42 patients were receiving long-term inhaled antibiotics and 129 were receiving long-term oral antibiotic treatments at baseline.

Baseline characteristics	Full cohort	Patients providing sputum
N	433	381
Age (years; mean- SD)	67 (58-74)	67 (58-74)
Gender (% female)	263 (60.7%)	225 (59.1%)
Body mass index	25.0 (22.3-28.5)	25.1 (22.2-28.6)
Smoking status (never/ex/current	266(61%)/151(34.9%)/ 16(3.7%)	239(62.7%)/131(34.4%) /11(2.9%)
MRC dyspnoea score	2 (1-3)	2 (1-3)
FEV_1 Litres	1.58 (1.10-2.20)	1.58 (1.10-2.23)
FEV_1 % predicted	71.9 (50.0-91.0)	71.4 (49.4-90.9)
FVC Litres	2.45 (1.84-3.21)	2.41 (1.85-3.19)
FVC % predicted	83.9 (68.4-99.5)	83.2 (67.7-98.7)
Aetiology of bronchiectasis - Idiopathic - Post-infective - Previous ABPA - Asthma - COPD	 195 (45.0%) 84 (19.4%) 37 (8.5%) 15 (3.5%) 22 (5.1%)	 169 (44.4%) 78 (20.5%) 34 (8.9%) 14 (3.7%) 19 (5.0%)

- Rheumatoid arthritis	21 (4.8%)	17 (4.4%)
- Connective tissue disease	6 (1.4%)	4 (1.0%)
- Inflammatory bowel disease	11 (2.5%)	11 (2.9%)
- Primary Immunodeficiency	18 (4.2%)	17 (4.5%)
- Previous NTM infection	7 (1.6%)	4 (1.0%)
- Primary ciliary dyskinesia	4 (0.9%)	3 (0.8%)
- Alpha 1 antitrypsin deficiency	2 (0.5%)	1 (0.3%)
- Others	11 (2.5%)	10 (2.6%)
Exacerbations per year	1 (0-3)	1 (0-3)
Prior hospitalisation for severe exacerbations	107 (24.7%)	101 (26.5%)
St Georges Respiratory questionnaire total score	44.3 (24.6-62.7)	46.1 (27.3-63.2)
Bronchiectatic on CT	3 (2-4)	3 (2-4)
Reiff Score	3 (2-6)	3 (2-6)
Chronic colonisation*	236 (54.5%)	213 (55.9%)
H. influenzae	129 (29.8%)	116 (30.4%)
P. aeruginosa	63 (14.5%)	60 (15.7%)
M. catarrhalis	51 (11.8%)	49 (12.9%)
S. pneumoniae	25 (5.8%)	23 (6.0%)
S. aureus	34 (7.9%)	30 (7.9%)
Enterobacteriaceae	39 (9.0%)	39 (10.2%)
Bronchiectasis severity index (mean	6 (4-10)	6 (4-11)
Mild	126 (29.1%)	108 (28.3%)
Moderate	170 (39.3%)	144 (37.8%)
Severe	137 (31.6%)	129 (33.9%)

Table 1. Baseline characteristics of the cohort. Data are presented as median (interquartile range) or N (%).

*defined as isolation of a pathogenic microorganism in sputum when clinically stable on 2 occasions at least 3 months apart in a 12 month period. Abbreviations: ABPA= allergic bronchopulmonary aspergillosis, FEV_1= forced expiratory volume in 1 second, FVC= forced vital capacity, MRC= Medical Research Council, NTM= non-tuberculous Mycobacteria.

Sputum neutrophil elastase activity is associated with disease severity

The ABI-NE assay detected activity above the lower limit of detection in 317 patients (83.2%) while the kinetic-NE assay detected active NE in 274 (71.9%) of samples. The two assay methods were highly correlated (Figure E1 online).

Sputum NE activity as measured by the ABI-NE assay showed a univariate association with cross-sectional markers of disease severity including MRC dyspnoea score ($r=0.34, p<0.0001$), SGRQ score ($r=0.28, p<0.0001$), absolute FEV_1 ($r=-0.31, p<0.0001$), FEV_1 % predicted ($r=-0.33, p<0.0001$), Reiff

score (r=0.29,p<0.0001), and the BSI (r=0.49,p<0.0001). Similar results were obtained with the kinetic-NE assay, Figure 2, and figure E2 online.

Both ABI-NE and kinetic-NE assays were correlated with sputum MPO activity (p<0.0001) but there was no correlation with sputum CXCL8, IL-1β, TNF-α or EN-RAGE (data not shown).

There was a relationship between NE activity and airway bacterial load (at bacterial load above 10(7) cfu/ml- figure 3A and 3B). Patients chronically infected with *P. aeruginosa,* enterobacteriaceae and *H. influenzae* had increased levels of NE using both assays (p<0.0001) compared to patients without chronic bacterial infection.

Sputum NE activity and longitudinal clinical outcomes

In the sputum producing cohort, the mortality rate was 8.7% and 25.5% of patients experienced hospital admissions for severe exacerbations during follow-up. The median frequency of exacerbations was 1 per patient per year (IQR 0-3).

As the ABI-NE assay was more sensitive and had stronger correlations with the majority of clinical outcomes for clarity we only present the results for the ABI-NE assay here. Using ROC analysis, ABI-NE activity was associated with hospital admissions during follow-up AUC 0.75 (0.72-0.79) and mortality AUC 0.70 (0.67-0.73). Entering NE activity as a continuous variable, after multivariable adjustment including the BSI, a 1µg/ml increase in NE activity was independently associated with a 0.5% increased risk of hospital admission (HR 1.005 95% CI 1.002-1.008,P<0.0001). No independent relationship with mortality was identified. Additional models are shown in table E1.

Using ROC analysis, we determined candidate clinically meaningful cut-off values of sputum NE. 132 patients had values below the lower limit of detection (<0.016μg/ml- low NE), 143 patients had values between 0.016 and 20μg/ml (intermediate NE), while 106 had values >20μg/ml (high NE).

Comparing the frequency of exacerbation between the three ABI-NE cut-offs using Poisson regression, patients with the highest elastase values (>20μg/ml) had a RR of 3.18 (95% CI 2.65-3.18,p<0.0001) and patients with intermediate NE values had a rate ratio (RR) of 1.61 (95%CI 1.39-1.86,p<0.0001) compared to the low elastase (reference) group indicating that high sputum NE activity was associated with a greatly increased frequency of future exacerbations. For severe exacerbations the corresponding values were RR 1.69 (95% CI 0.90-3.17,p=0.1) for intermediate NE values and 4.73 (2.67-8.33,p<0.0001) for the highest NE group- Figure 4A. Consistent with this, elevated NE activity was associated with a shorter time to next exacerbation (p<0.0001, Figure 4B), shorter time to next severe exacerbation (p<0.0001,Figure 4C) and increased all-cause mortality (Figure 4D,p<0.0001). An analysis of exacerbation frequency according to different severity groups using the BSI is shown in table E2 online. Prediction statistics are shown in table E3 online.

FEV_1 decline over 3 years was normally distributed. The mean FEV_1 decline was 48.2mls per year (standard deviation 83.7). Across the defined 3 elastase groups, mean FEV_1 decline was 35.6mls (sd 81.1) for NE activity <0.016μg/ml, 49.5ml (sd 92.5) for intermediate elastase levels and 56.4ml (sd 67.4) for those with NE >20μg/ml. On univariate regression there was a weak but statistically significant relationship between NE activity and FEV_1 decline (p=0.004). After adjustment for BSI, gender and baseline FEV_1, increasing NE-ABI elastase was associated with more rapid lung function decline (beta coefficient -0.139,p=0.001, model fit r=0.7).

Serum desmosine is associated with age and disease severity

cDES was most strongly correlated with age (r=0.48,p<0.0001, Figure 5A). Additional univariate correlations were observed between cDES and MRC dyspnoea score (r=0.32,p<0.0001), SGRQ (r=0.40,p<0.0001), absolute FEV_1 (r=-0.39,p<0.0001) and Reiff score (r=0.15,p=0.002). cDES was also significantly higher in patients colonised with *P. aeruginosa* (p<0.0001). There was an association between cDES and BSI (r=0.46,p<0.0001). Correlations were demonstrated with sputum NE activity (Figure E1 online). Removing the outliers at >1ng/ml showed similar correlations with markers of disease severity as described in the online supplement (p7).

In the total cohort, mortality was 9.5% and 22.6% of patients were admitted to hospital for severe exacerbations. Median exacerbation frequency was 1 per patient per year (IQR 0-3).

In analysis of longitudinal clinical outcomes, there was no relationship between cDES and FEV_1 decline over 3 years (p=0.1), but there was a strong relationship between cDES and severe exacerbations (HR 6.0 95%CI 3.61-10.0,p<0.0001) which persisted after adjustment for BSI (HR 2.7 95% CI 1.42-5.29,p=0.003). There was no significant association between cDES and moderate exacerbations (p=0.2), but after combining moderate and severe exacerbations a statistically significant association above 0.4ng/ml was observed – RR 1.96 95% CI 1.61-2.39,p<0.0001.

There was similarly an association between cDES and all-cause mortality (HR 2.60 95% CI 1.24-5.45,p=0.01), but this relationship was not statistically significant after adjustment for BSI (HR 1.15 95% CI 0.45-2.91,p=0.8). Additional models are shown in table E2. The AUC values for biomarkers compared to individual recognised predictors of outcome in bronchiectasis is shown in table E4.

A sensitivity analysis conducted in patients taking long term antibiotics demonstrated that sputum NE and cDES had similar associations with long term outcomes compared to the overall cohort (table E5).

Changes in neutrophil elastase at exacerbation and after antibiotic therapy

To determine whether sputum NE was responsive to treatment we studied 26 patients during an acute exacerbation requiring intravenous antibiotic therapy. Characteristics of the patients included compared to the overall population are shown in table E6.

Median ABI-NE levels were 0.39µg/ml (IQR 0-23.5) at baseline, 57.0µg/ml (3.3-145µg/ml) at onset of exacerbation, 0µg/ml (0-25.8) after 14 days of antibiotic therapy and 1.3µg/ml (0-29.9) at the second stable measurement 6 months later (Figure 6). Although NE activity was generally higher at exacerbations than at baseline ($p=0.0002$) and at recovery ($p<0.0001$), the assay did not discriminate between exacerbation and disease quiescence because of the high baseline activity in some individuals. The ABI-NE assay level >50µg/ml was associated with a sensitivity of 57.7% and specificity of 92.3%. An increase from baseline was present at exacerbation in 20/26 patients at exacerbation.

Remarkably, even with this small sample size, failure to return to NE baseline levels after completion of antibiotics was associated with a shorter time to the next exacerbation (HR 2.92, 95% CI 1.16-7.38,$p=0.02$). Data on the correlation between elastase measurements at two stable visits > 6months apart are shown in Figure E3 online.

Discussion

This study indicates a role for sputum NE activity as a biomarker of disease severity and disease progression in bronchiectasis, while also providing the first data on the linked biomarker cDES. NE activity in sputum was independently associated with risk of exacerbations, severe exacerbations and lung function decline, even after adjustment for underlying severity of the disease. This suggests NE is a useful marker that may identify patients at future risk. Elastase is dynamic, responding to treatment and we show that a failure to improve elastase with treatment predicts a shorter time to next exacerbation. To the best of our knowledge, NE activity is the first biomarker to be associated with this range of clinically relevant outcomes in bronchiectasis. This confirms and extends previous observations in diverse bronchiectasis populations in Hong Kong, Belgium and the UK. (13-15)

Tsang *et al* previously showed that 24h NE output correlated with 24h sputum volume, radiological severity of bronchiectasis and FEV_1.(13) NE is not the only airway protease found in the bronchiectasis lung, but Goeminne *et al* in a study of 63 patients, showed that NE accounted for 82% of the total gelatinolytic activity of sputum, making a greater contribution than matrix metalloproteinases.(15) Goeminne *et al* also showed a statistically significant association between NE and FEV_1% predicted, which was not seen for MMP-9.(15) In the largest previous study on 385 patients with bronchiectasis, sputum NE activity measured using a kinetic assay was found to be associated with bacterial load, *P. aeruginosa* infection, radiological severity and lung function.(14) Previous studies, however, have used a variety of different assays, and a limited number of bronchiectasis severity indices without longitudinal follow-up.

It is essential that candidate biomarkers undergo independent validation because markers typically perform better in their “discovery” or derivation cohort than in subsequent independent cohorts.(28) Our study therefore validates these previous findings in a large cohort, as we demonstrate a clear association between elastase activity and a variety of markers of disease severity including breathlessness, quality of life and FEV_1. There was a strong relationship between elastase activity and the multidimensional BSI.(20)

We observed strong relationships between NE activity and bacterial load. NE activity was also highest in patients with *P. aeruginosa* infection and this is consistent with previous studies in bronchiectasis which have shown that bacteria, and *P. aeruginosa* in particular, are the key drivers of airway neutrophilic inflammation (22), and that that *P. aeruginosa* infection represents a distinct clinical phenotype associated with earlier mortality, more frequent exacerbations and worse quality of life.(14, 22, 29, 30)

While a biomarker that identifies patients with more severe disease is of interest, it is most important to find biomarkers that can identify patients at highest risk of future exacerbations and disease progression. Our study shows that NE activity is independently associated with lung function decline over 3 years, identifying elastase as the first biomarker that is associated with disease progression in bronchiectasis. In addition, elastase was an independently associated with future exacerbations. Although patients with the highest elastase levels were at an increased risk of early death, this association was not independent of disease severity using the BSI, and only the highest levels of elastase were associated with increased mortality, indicating that airway inflammation itself was not likely to be the primary driver of mortality in this population.

Our data are consistent with those seen in CF, where neutrophil elastase has been shown to be a key biomarker.(31-33) In a pooled analysis of 4 multicentre studies Mayer-Hamblett et al, showed a clear relationship between elastase and FEV_1.(31) Sagel *et al* extended these observations demonstrating that elastase was the strongest predictor of lung function decline over 3 years.(32) Sly also demonstrated that elastase activity present in BAL was the strongest predictor of the early development of bronchiectasis in infants with CF.(33)

Bronchiectasis has been a neglected disease and so, in contrast, biomarker studies are in their infancy. Markers identified in bronchiectasis include sputum MMP-8 and MMP-9 which were shown in a Chinese cohort to correlate with radiological severity, FEV_1 and the BSI. (34) These findings were extended by Taylor *et al*, who showed that MMP-8 and MMP-9 were higher with *P. aeruginosa* or *H.*

influenzae colonisation and inversely associated with lung function.(34,35) These studies included 102 and 86 patients respectively and included only 1 year of follow-up, therefore further large validation studies are required.

Inconsistent results were seen with cytokines such as CXCL8, TNF-α and IL-1β in previous studies and in the present study these were not significantly associated with clinical outcomes.(14, 36)

No blood biomarkers have been studied in detail in bronchiectasis which makes the identification of cDES as a potential marker of severity of significant interest. The results here are similar to those recently described in a large COPD cohort where cDES was associated with age and quality of life. (17)

There is overwhelming evidence that elastase is involved in the pathophysiology of bronchiectasis. Destruction of elastin, basement membrane collagen and proteoglycans by proteases contributes to disease progression and may explain the relationship between elastase and FEV_1 decline observed in this study.(37) NE induces neutrophil dysfunction through multiple mechanisms including cleavage of FcγRIIIb, and has also been shown to cleave complement receptor 1 in patients with CF.(3, 38) NE can also cleave the opsonin iC3b from the surface of pathogens, leading to opsonin/receptor mismatch(39) while Vandivier *et al* showed that elastase cleaved phosphatidylserine, preventing the phagocytosis and clearance of apoptotic cells. (40) Not surprisingly therefore, therapeutic manipulation of elastase has been proposed in bronchiectasis. In a proof of concept study, Stockley *et al* tested an oral NE inhibitor for 4 weeks in 38 patients with bronchiectasis. (41) Although the primary outcome of a reduction in sputum neutrophils was not achieved, the study showed a clinically important and significant improvement in FEV_1 of 100ml vs placebo, and a greater than 4-point improvement in the SGRQ which did not reach statistical significance.(41)

While NE activity appears able to stratify patients as high and low risk of disease progression, we cannot currently recommend management decisions based on elastase measurement. The next step would be implementation of such a strategy in a controlled clinical trial. Sputum biomarkers are not

currently in routine use, and implementation would be greatly enhanced by the availability of a point of care device that could make the assay more rapid and accessible.

As we and others have shown that elastase is responsive to change and that changes in elastase correlate with clinical outcomes, measurement of NE may be particularly useful in clinical trials, where it could be used as an early "signal searching" or "early efficacy" end-point for new antibiotics or anti-inflammatory therapies.(14, 42) Such end-points are essential to identify candidates in smaller clinical trials before embarking on large definitive phase-3 studies. It has been suggested that the absence of such an early response end-point has contributed to the failure of a number of large phase-3 programmes to reach their primary end-points.(43) NE should be further evaluated for this purpose.

There are a wide range of NE assays commercially available and our data only demonstrate the validity of the ABI-NE, kinetic NE and cDES assays in bronchiectasis. Additional studies with alternative assays including those quantifying total elastase rather than elastase activity and urinary desmosine may not give the same results. This study is single centre, although the external validity of our results are strengthened by the similarity of the characteristics of these patients with other cohorts across Europe and the previous validation of findings from our centre across multiple European centres. (20, 44) We did not obtain multiple elastase measurements over time except in a subset and it will be important in future to determine if repeated measurement of elastase could provide improved predictive accuracy. The cut-offs that we proposed here for intermediate and high levels of NE have not been independently validated and should be tested in future cohort studies. The use of expectorated sputum for NE measurement may introduce sampling bias because only patients able to produce sputum could be included.

Conclusion

Sputum NE activity is associated with the future risk of exacerbations, including severe exacerbations and lung function decline in bronchiectasis. Elastase is therefore a marker of disease progression in bronchiectasis that may complement clinical assessment of multidimensional clinical scoring systems. NE levels reflect clinical status, and its response is associated with future risk of exacerbations. Future interventional studies should therefore evaluate whether elastase reduction can be used as a surrogate of efficacy in clinical trials.

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Figure legends

Figure 1. STROBE flow chart of study inclusion and exclusions. Abbreviations: NTM= non-tuberculous mycobacteria, ABPA= allergic bronchopulmonary aspergillosis, Bx= bronchiectasis, CT= computed tomography, ABI-NE= Activity based immunoassay for neutrophil elastase. Patients with elastase levels below the lower limit of detection "not detected" were included in the analysis with values treated as zero.

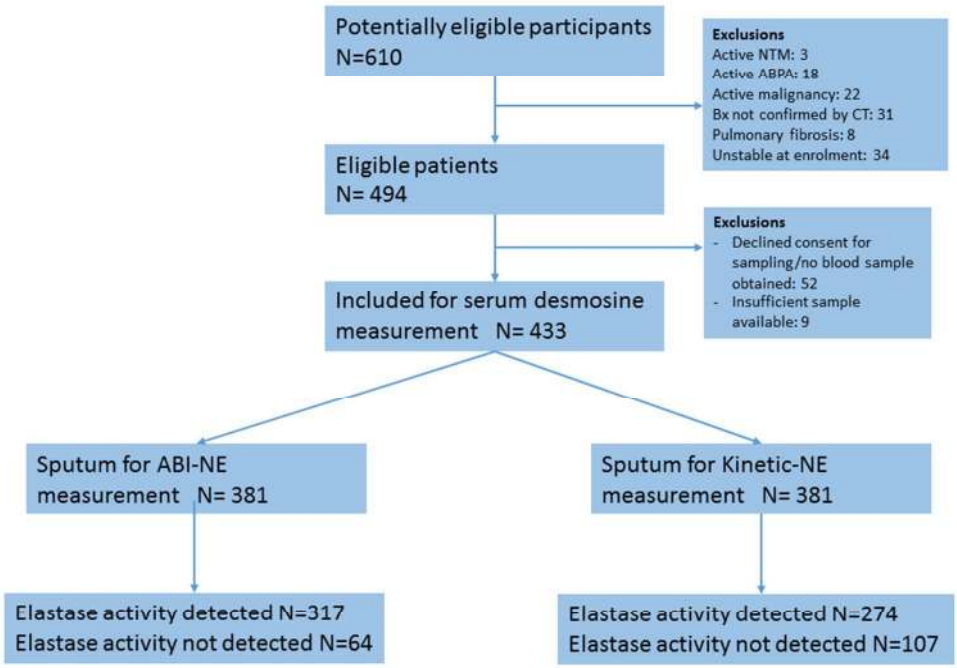
Figure 2. Association between neutrophil elastase and severity of disease. The ABI-NE assay (upper panels) and kinetic NE assay (lower panels) are significantly different between bronchiectasis severity index and FEV_1 % predicted groups and correlate with the SGRQ. NS= no significant difference compared to FEV_1 >80% predicted. *p<0.05 compared to >80% predicted FEV_1 ***p<0.0001 compared to >80% FEV_1 % predicted. Across group comparisons for FEV_1 p<0.0001 by Kruskal-Walis test. BSI and FEV1 cut-offs were chosen as those used in (20) Error bars show median with interquartile range.

Figure 3. Association between neutrophil elastase and sputum bacterial load. Figure 3A shows data for the ABI-NE assay, 3B shows the Kinetic assay. ***p<0.0001 compared to no organism isolated. Figures show median with the upper error bar showing the upper limit of the interquartile range.

Figure 4. Association between neutrophil elastase and longitudinal clinical outcomes. Figure 4A Odds ratios from Poisson regression, elastase level >0.016µg/ml are associated with significantly increased risk of moderate exacerbations (p<0.0001) and levels >20µg/ml are associated with increased severe exacerbations (p<0.0001). B: Elevated neutrophil elastase is associated with shorter time to next exacerbation (p<0.0001 by Log rank test), C: Time to next hospitalisation for severe exacerbation over 36 months (p<0.0001 by log rank test), D: All-cause mortality over 3 years (p<0.0001 by log rank test, comparison between <0.016µg/ml vs 0.016-20µg/ml= NS). <0.016µg/ml, n=132, 0.016 µg/ml-20 µg/ml,n=143 and >20 µg/ml, n=106 patients.

Figure 5. Spearman rank correlation analysis of the relationship between serum total desmosine and age, bronchiectasis severity index (BSI), SGRQ and FEV_1 % predicted.

Figure 6. Changes in sputum NE activity at exacerbation and recovery.



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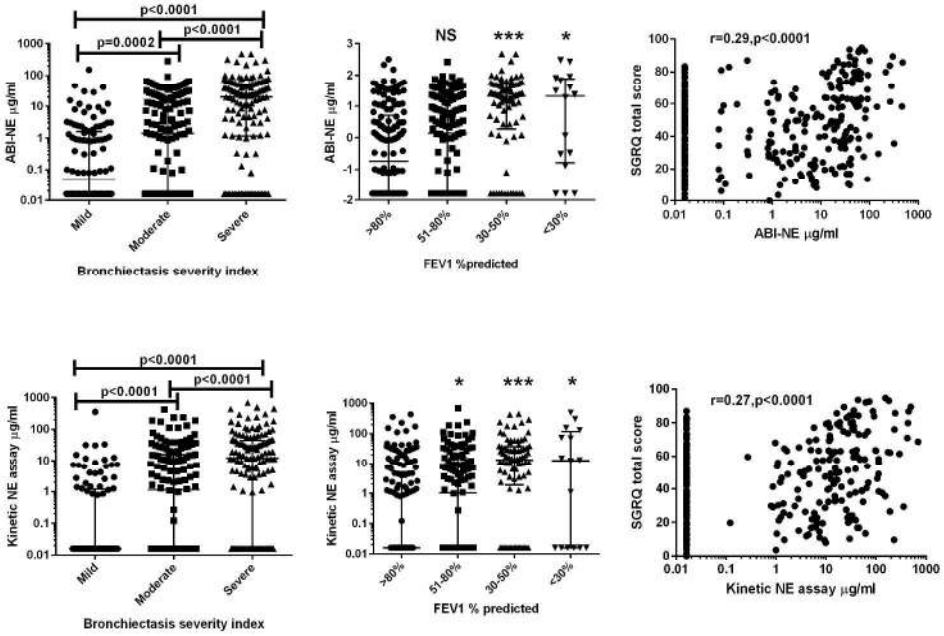

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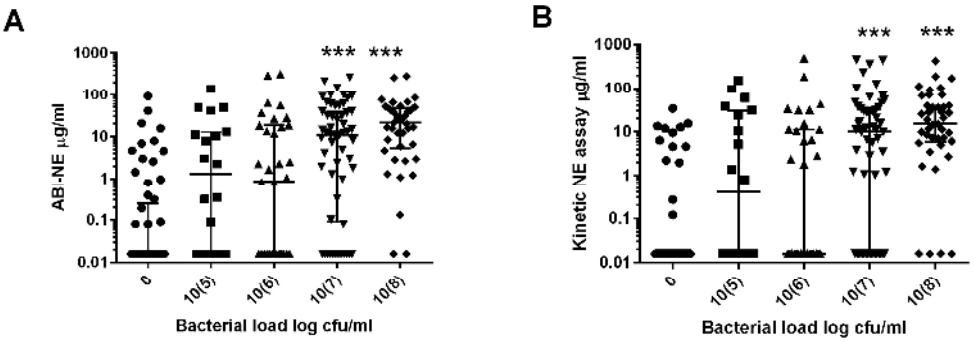

Figure 3. Association between neutrophil elastase and sputum bacterial load. Figure 3A shows data for the ABI-NE assay, 3B shows the Kinetic assay. ***p<0.0001 compared to no organism isolated. Figures show median with the upper error bar showing the upper limit of the interquartile range.

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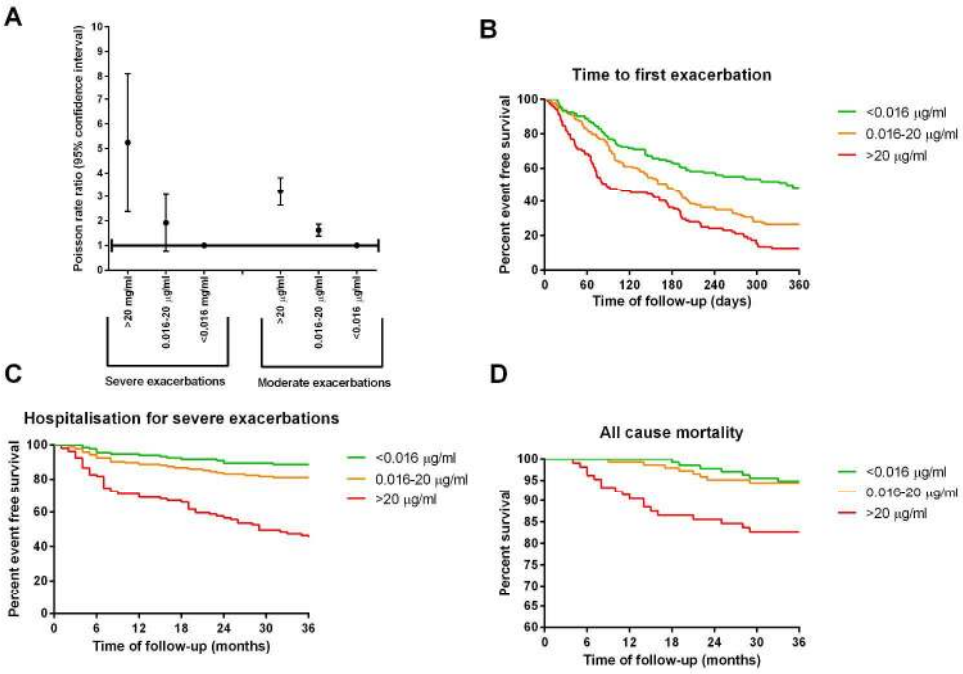

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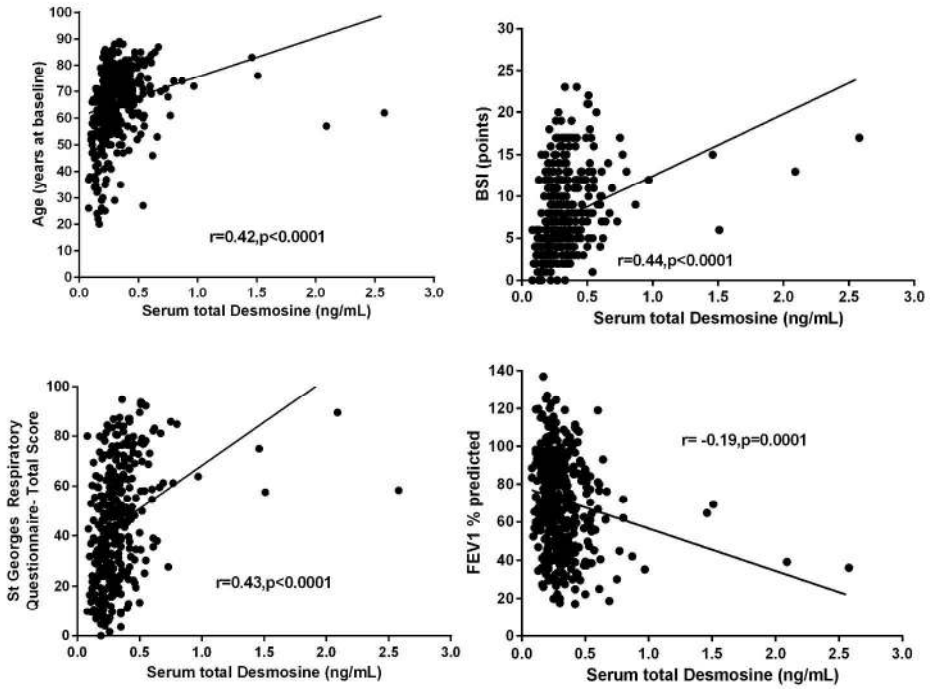

Figure 5. Spearman rank correlation analysis of the relationship between serum total desmosine and age, bronchiectasis severity index (BSI), SGRQ and FEV1 % predicted.

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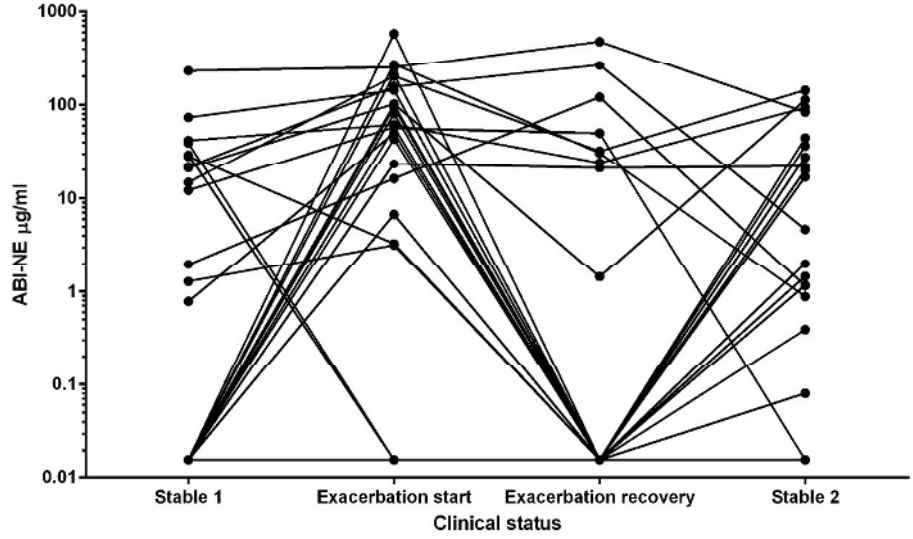

Figure 6. Changes in sputum NE activity at exacerbation and recovery.

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Online supplementary material for:

Neutrophil elastase activity predicts exacerbations and lung function decline in bronchiectasis

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Supplementary Methods

Clinical assessments

Patients were managed at a specialist bronchiectasis clinic and received investigations and management in line with the recommendations of the British Thoracic Society (BTS) bronchiectasis guidelines.(19) Aetiology was determined by standardised testing according to BTS recommendations and performed in all patients through a standardised computer "order set". (19) The bronchiectasis severity index was calculated as previously described. (20) Sputum was sent for standard qualitative and quantitative microbiology. (14) The study was initiated prior to the published validation of the Quality of Life Bronchiectasis Questionnaire and so quality of life was evaluated using the St. Georges Respiratory Questionnaire (SGRQ), the most widely used quality of life tool in bronchiectasis research at that time. (21) Cough was measured with the Leicester cough questionnaire. Breathlessness was quantified by the MRC dyspnoea score. Chronic colonisation was

defined as the isolation of pathogens on at least 2 occasions 3 months apart during the preceding 12 months. (22) The predominant pathogen is defined as the organism isolated most frequently during the study period. Spirometry was performed according to ERS guidelines. (23) The severity of radiological bronchiectasis was evaluated using the Reiff score, which is a simple classification system that grades the degree of dilatation per lobe (considering the Lingula as a separate lobe) and awards points as follows 1=tubular/cylindrical bronchiectasis 2= varicose bronchiectasis 3= cystic bronchiectasis. (24) The maximum score is 18 points.

Patient data was linked using a unique identifier to electronic medical records systems available in the East of Scotland allowing documentation of all antibiotic prescriptions, hospital admissions and all-cause mortality. This was used to validate investigator recorded data. In the case of exacerbations, primary care prescriptions were used to confirm self-reported exacerbations.

Comparison of methods for quantification of neutrophil elastase activity in bronchiectasis

The ProteaseTag® Active NE activity-dependent immunoassay (ProAxsis Ltd.) was carried out according to the manufacturer's instructions (http://proaxsis.com/products_list/neutrophil-elastase). Briefly, active NE activity is selectively captured from the sample using a specific NE-Tag and the subsequent antibody step provides additional signal amplification with increased sensitivity. The kinetic assay was conducted using the fluorogenic substrate, *N*-Methoxysuccinyl-Ala-Ala-Pro-Val-7-amido-4-methylcoumarin (Sigma-Aldrich) at a final concentration of 50 μM in 50 mM Tris/HCl buffer, pH 7.7 containing 0.1 M NaCl and hydrolysis monitored using an Optima FluoStar (BMG Labtech, Germany) at excitation 380 nm and emission 460 nm. In each case, a calibration curve was constructed using active native NE (Merck Millipore). Neutrophil elastase data were non-normally distributed and so data are presented as median with interquartile range (IQR). Across all patients, the ABI-NE assay found a median of 1.67μg/ml (IQR 0-23.9) while the kinetic NE assay showed a median of 1.25 μg/ml (0-18.7).

The NE-ABI detected neutrophil elastase activity above the lower limit of detection in 317 patients (83.2%) while the kinetic assay detected active NE in 274 (71.9%) of samples. The two assay methods were highly correlated (Figure E1A) but NE-ABI showed superior sensitivity identifying active NE in 44 samples where it was undetectable with the kinetic assay. Conversely there was 1 sample where active NE was detected by the kinetic assay where it was undetectable with the NE-ABI. The Bland-Altman plot showed good agreement between the two sputum NE assay methods.

Median serum desmosine levels were 0.28ng/ml (IQR 0.21-0.36). There was a significant correlation between serum desmosine level and NE-ABI (Figure E1C) and also the kinetic-NE assay (Figure E1D)

Statistical analysis- supplementary information

Mean and standard deviation were used to display continuous normally distributed data with median and interquartile range for continuous non-normally distributed data, and frequencies and percentages for categorical data. Distribution was evaluated with the D'Agostino-Pearson Omnibus test unless otherwise indicated. The association of biomarkers with linear variables e.g other assays and quality of life, was performed using Spearman correlation which does not assume a normal distribution and assesses the strength and direction of a monotonic relationship whether linear or not. For data organised into groups, such as the mild, moderate and severe categories of the bronchiectasis severity index or the established cut-offs for FEV1, between group differences were evaluated by ANOVA or Kruskal Wallis test according to the distribution of the data. Frequency of exacerbations and severe exacerbations were evaluated using Poisson regression adjusted for duration of follow-up. Time to event data (time to first exacerbation, first hospital admission and death) were analysed using Kaplan-Meier survival analysis for unadjusted data and Cox proportional hazard regression for multivariable analyses. Time zero for these analyses was the date of elastase or desmosine measurement as appropriate with follow-up until death, censor (loss to follow-up) or end of study at 3 years. For the analysis of time to next exacerbation in the exacerbation subcohort,

time zero was day 14 post exacerbation. Discrimination for mortality and severe exacerbations at 3 years was analysed using the area under a receiver operator characteristic curve (AUC). For these analyses, a fixed time point of 3 years was used. Analysis of FEV1 decline over 3 years follow-up was performed using multivariable linear regression with appropriateness of the linear regression modelling evaluated by examining the distribution of residuals. In some analyses, patients were split into 3 groups based on low, intermediate and high elastase levels. Cut-offs were determined by analysis of the receiver operator characteristic curves with candidate cut-offs selected using Youden's index. Patients with missing data were excluded from analysis of the specific test as outlined below. No imputation methods were used. Missing follow-up data was minimised through the use of electronic data. Sample size was empirical based on previous studies with equivalent lengths of follow-up. (14)

Analyses were conducted using SPSS Version 21 for Windows (SPSS, Chicago, Illinois, USA) and Graph Pad Prism Version 6 (Graph Pad Software, Inc. San Diego, California, USA).

Supplementary Results, Figures and Tables

Supplementary data are presented in the order in which they appear in the text.

Comparison between different methods for evaluating sputum elastase activity and serum desmosine.

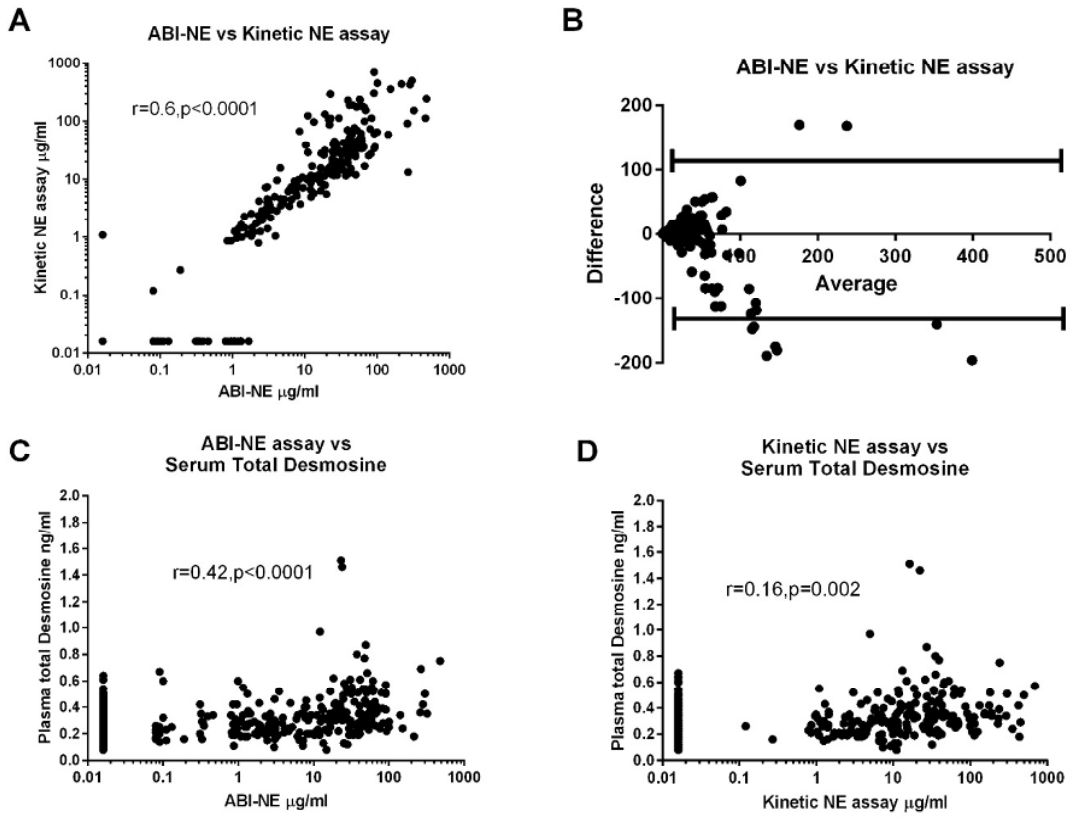

Figure E1. Comparison of 3 methods for quantification of neutrophil elastase activity in BE. A: comparison of two methods of quantifying sputum neutrophil elastase activity by linear regression, B: Bland-Altman comparison between the two methods of sputum neutrophil elastase quantification C: Correlation between activity based immunoassay for neutrophil elastase (ABI-NE) and serum total desmosine. D: correlation between activity determined by kinetic-NE assay and serum total desmosine.

Relationship between sputum neutrophil elastase activity and disease severity and activity using the kinetic assay

Using the kinetic elastase activity assay, there were statistically significant relationships between elastase and MRC dyspnoea score (r=0.33,p<0.0001), Leicester cough questionnaire (r=-0.23,p<0.0001), SGRQ (r=0.27,p<0.0001), absolute FEV_1 (r=-0.30,p<0.0001), FEV_1 % predicted, (r=-0.33,p<0.0001), Reiff score (r=0.29,p<0.0001), and BSI (r=0.48,p<0.0001).

Both assays were strongly associated with sputum colour using the Murray chart as shown in figure E2 below.

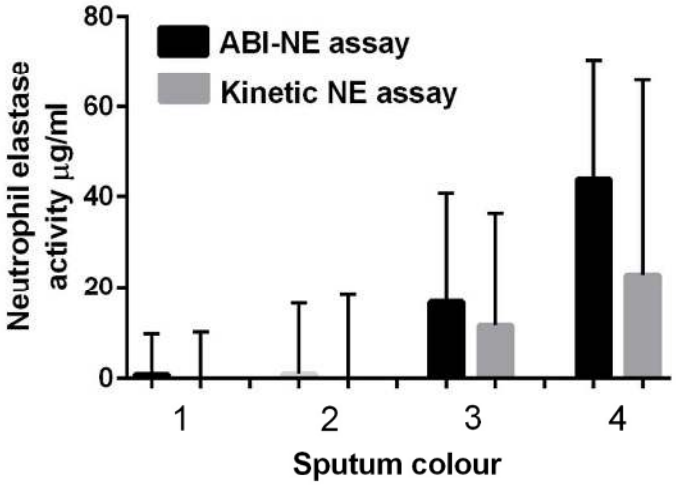

Figure E2. Relationship between NE activity using two different assays and sputum colour. Comparisons across the groups are statistically significant p<0.0001 by Kruskal-Walis test for both assays. Sputum colour was assessed using the Chart of Murray et al (ERJ 2011).

Table E1. Additional models evaluating the association between NE activity and mortality or hospital admissions

Assay and model	Mortality	Hospital admissions
ABI-NE		
Unadjusted	1.04 (1.00-1.07),p=0.04	1.09 (1.07-1.11),p<0.0001
Model 1	1.04 (1.00-1.08),p=0.04	1.09 (1.07-1.11),p<0.0001
Model 2	1.00 (0.92-1.03),p=0.5	1.05 (1.02-1.08),p<0.0001
Model 3	1.00 (0.92-1.03),p=0.4	1.05 (1.02-1.08),p=0.001
Kinetic NE		
Unadjusted	1.04 (1.02-1.06),p<0.0001	1.03 (1.02-1.05),p<0.0001
Model 1	1.03 (1.01-1.05),p=0.003	1.03 (1.01-1.05),p<0.0001
Model 2	1.01 (0.98-1.03),0.5	1.01 (0.99-1.02),p=0.1

Model 3	1.01 (0.97-1.03),p=0.9	1.01 (0.99-1.02),p=0.1
Desmosine		
Unadjusted	2.60 (1.24-5.45),p=0.01	6.0 (3.61-10.0),p<0.0001
Model 1	2.35 (0.90-6.17),p=0.08	5.73 (3.37-9.76),p<0.001
Model 2	1.15 (0.45-2.91),p=0.8	2.7 (1.42-5.29),p=0.003
Model 3	10.5 (0.29-3.94),p=0.9	1.80 (0.90-3.59),p=0.1

Table E1. Summary of multivariable models. Model 1- adjusted for age and gender only, model 2- adjusted for BSI which incorporates age, MRC dyspnoea score, exacerbation frequency, prior hospitalisation, radiological severity, bacterial colonisation, *P. aeruginosa* status and BMI. Model 3- adjusted for age, gender, MRC dyspnoea score, exacerbation frequency, prior hospitalisation, radiological severity and *P. aeruginosa* as independent variables.

Table E2- Prediction of exacerbations independent of the Bronchiectasis severity index

Moderate and severe exacerbations	Low elastase group	Intermediate elastase group	High elastase group
BSI low risk	1.0 (reference)	1.42 (0.56-3.61),p=0.5	1.62 (1.08-2.45),p=0.02
BSI intermediate risk	1.0 (reference)	1.78 (0.91-3.46),p=0.09	1.84 (1.01-3.32),p=0.04
BSI high risk	1.0 (reference)	1.81 (1.36-2.41),p<0.0001	2.00 (1.53-2.63),p<0.0001
Moderate exacerbations	Low elastase group	Intermediate elastase group	High elastase group
BSI low risk	1.0 (reference)	1.46 (0.57-3.71),p=0.4	1.67 (1.10-2.52),p=0.02
BSI intermediate risk	1.0 (reference)	1.84 (0.95-3.58),p=0.07	1.87 (1.03-3.37),p=0.04
BSI high risk	1.0 (reference	1.80 (1.27-2.55),p=0.001	1.87 (1.34-2.60),p<0.0001

Table E2. Poisson regression analysis of different elastase groups demonstrating the independent predictive value of sputum elastase activity for exacerbations adjusted for the Bronchiectasis severity index.

Table E3- Predictive indices for NE activity and mortality or severe exacerbations

Organisms	PLR	NLR	Sensitivity	Specificity	PPV	NPV
Mortality						
ABI NE >20 µg/ml	2.2 (1.5-3.1)	0.6 (0.4-0.9)	54.6% (36.4-71.9%)	74.7% (69.8-79.2%)	17.0% (10.4-25.5%)	94.6% (91.2-96.9%)
ABI NE <0.016µg/ml	1.2 (1.0-1.5)	0.6 (0.3-1.2)	78.8% (61.1-91.0%)	35.9% (30.9-41.2%)	10.4 (6.9-14.9%)	94.7% (89.4-97.8%)
Severe exacerbations						
ABI NE >20 µg/ml	3.2 (2.3-4.3)	0.5 (0.4-0.7)	56.7% (46.3-66.7%)	82.0% (77.1-86.3%)	51.9% (42.0-61.7%)	84.7% (80-88.8%)
ABI NE <0.016µg/ml	1.4 (1.3-1.6)	0.4 (0.2-0.6)	84.5% (75.8-91.1%)	41.2% (35.4-47.2%)	32.9% (27.1-39.2%)	88.6% (81.9-93.5%)
Mortality						
Kinetic NE >20 µg/ml	1.9 (1.2-2.9)	0.7 (0.6-1.0)	42.4% (25.5-60.8%)	77.6% (72.8-81.9%)	15.2% (8.6-24.2%)	93.4% (89.9-96.0%)
Kinetic NE <0.016µg/ml	1.4 (1.1-1.8)	0.6 (0.3-1.0)	72.7% (54.5-86.7%)	48.3% (42.9-53.7%)	11.8% (7.7-17.0%)	94.9% (90.6-97.7%)
Severe exacerbations						
Kinetic NE >20 µg/ml	2.6 (1.8-3.6)	0.7 (0.6-0.8)	44.3% (34.2-54.8%)	82.8% (77.8-86.9%)	46.7% (36.3-57.4%)	81.3% (76.3-85.6%)
Kinetic NE <0.016µg/ml	1.9 (1.6-2.2)	0.3 (0.2-0.5)	81.4% (72.3-88.6%)	56.0% (50.0-61.9%)	38.7% (32.0-45.8%)	89.8% (84.4-93.9%)

Table E3. Predictive indices of NE cut-offs to predict mortality and hospital admissions for severe exacerbations.

Sensitivity analysis of desmosine without outliers

It was noted that 4 data points with circulating desmosine >1ng/ml were outliers that could have a significant impact on correlations with markers of disease severity. After excluding these outliers correlations were as follows: MRC dyspnoea score ($r=0.31, p<0.0001$), age ($r=0.37, p<0.0001$), LCQ ($r=-0.20, p=0.0002$), SGRQ ($r=0.39, p<0.0001$), absolute FEV1 ($r=-0.38, p<0.0001$, FEV1 % predicted ($r=-0.26, p<0.0001$) and reiff score ($r=0.14, p=0.003$).

Table 4 shows the AUC values for mortality and hospital admissions relative to recognised predictors of poor outcome in BE.

	Mortality prediction	Hospital admissions
ABI-NE	0.70 (0.67-0.73)	0.75 (0.72-0.79)
Kinetic-NE	0.66 (0.56-0.76)	0.74 (0.68-0.80)
Desmosine	0.72 (0.65-0.79)	0.65 (0.59-0.71)
Age	0.75 (0.67-0.83)	0.51 (0.45-0.58)
FEV1	0.72 (0.64-0.81)	0.72 (0.66-0.78)
Exacerbation freq.	0.67 (0.57-0.76)	0.79 (0.74-0.85)
MRC dyspnoea score	0.66 (0.56-0.75)	0.79 (0.74-0.84)
P. aeruginosa	0.56 (0.46-0.66)	0.72 (0.65-0.79)
Lobes involved on CT	0.60 (0.52-0.68)	0.66 (0.61-0.72)
BSI score	0.78 (0.70-0.84)	0.89 (0.85-0.93)

Table E4. Comparison of different individual clinical predictors of outcome in bronchiectasis. Abbreviations BMI= body mass index, FEV1= forced expiratory volume in 1 second, BSI= bronchiectasis severity index.

Table E5. Sensitivity analysis in patients using long term antibiotic treatments

Assay	Mortality (HR)	Hospitalisation (HR)
Long term antibiotic users		
ABI-NE	1.02 (0.98-1.06),p=0.3	1.06 (1.04-1.09),p<0.0001
Kinetic-NE	1.04 (1.01-1.06),p=0.003	1.02 (1.00-1.04),p=0.01
Desmosine	1.91 (0.65-5.56),p=0.2	3.72 (1.86-7.44),p<0.0001
Long term oral antibiotics only		
ABI-NE	1.02 (0.96-1.09),p=0.5	1.09 (0.98-1.20),p=0.1
Kinetic-NE	1.03 (0.99-1.08),p=0.1	1.02 (0.99-1.05),p=0.2
Desmosine	1.79 (0.44-7.25),p=0.4	3.59 (1.68-7.65),p=0.001
Long term inhaled antibiotics		
ABI-NE	1.36 (1.12-1.62),p=0.004	1.16 (1.06-1.27),p=0.001
Kinetic-NE	1.03 (1.00-1.06),p=0.03	1.02 (1.00-1.04),p=0.04
Desmosine	21.7 (0.40-1246.2),p=0.8	4.03 (1.49-10.91),p=0.006

Table E5. Association of biomarkers with clinical outcomes in patients receiving long term antibiotic treatments.

We also studied the association with exacerbations in these groups. In patients receiving long term inhaled antibiotics we observed a RR 3.18 (2.65-3.83),p<0.0001 for the highest elastase group and 1.61 (1.39-1.86),p<0.0001 for the intermediate elastase levels compared to the low elastase group (reference). In patients receiving oral antibiotics only, the corresponding values were 1.47 (1.11-1.95),p=0.007 and 1.28 (1.02-1.60),p=0.03 respectively. For patients receiving any long term antibiotic the effect was 2.04 (1.57-2.65),p<0.0001 and 1.36 (1.12-1.63),p=0.002 respectively compared to the reference group (NE <0.016ug/ml).

Table E6. Characteristics of patients in the exacerbation substudy

The patients included in the severe exacerbation substudy were predominantly idiopathic bronchiectasis and non-smokers. Of 26 exacerbations, at diagnosis of exacerbation, sputum culture showed *P. aeruginosa* in 14 (54%), *H. influenzae* in 5 (19%), *M. catarrhalis* and *S. aureus* both in 2 patients (8%) and *E.coli, S. pneumoniae* and *S.maltophilia* in 1 patient each. Compared to the overall study population, those with severe exacerbations had more severe disease in terms of worse radiological scoring, lower BMI, more frequent exacerbations and a higher bronchiectasis severity index score.

	Exacerbation sub-cohort	Patients not included in the exacerbation cohort	Overall cohort (sputum producers)	p-value
N	26	355	381	
Age (years)	68 (60-73)	67 (58-74)	67 (58-74)	0.8
Gender (% female)	16 (61.5%)	209 (58.9%)	225 (59.1%)	0.8
Aetiology				
Idiopathic	12 (46.2%)	157 (44.2%)	169 (44.4%)	0.9
Post-infective	6 (23.1%)	72 (20.3%)	78 (20.5%)	0.7
Others	8 (30.8%)	126 (34.5%)	134 (35.2%)	0.6
Smoking (never)	22 (84.6%)	217 (61.1%)	239 (62.7%)	0.02
MRC dyspnoea score	3 (2-4)	2(1-3)	2 (1-3)	0.4
Reiff score	8 (5-10)	3 (2-6)	3 (2-6)	<0.0001
SGRQ total score	58.3 (30.2-72.8)	45.6 (26.9-63.0)	46.1 (27.3-63.2)	0.1

FEV1% predicted	65.7 (42.1-85.6)	72.2 (50.7-91.5)	71.4 (49.4-90.9)	0.2
FVC % predicted	75.6 (55.9-95.4)	83.9 (68.5-99.3)	83.2 (67.7-98.7)	0.2
BMI	23.6 (20.7-26.4)	25.2 (22.3-28.9)	25.1 (22.2-28.6)	0.01
Annual exacerbations	3 (1-4)	1 (0-3)	1 (0-3)	0.005
BSI total score	12 (7-16)	6 (4-10)	6 (4-11)	<0.0001

Table E6. Characteristics of patients included in the exacerbation substudy. Abbreviations MRC=medical research council, SGRQ= St.Georges Respiratory Questionnaire, FEV1= forced expiratory volume in 1 second, FVC= forced vital capacity, BMI= body mass index, BSI= bronchiectasis severity index. p-values refer to comparisons between exacerbation subcohort and those not included in the exacerbation subcohort.

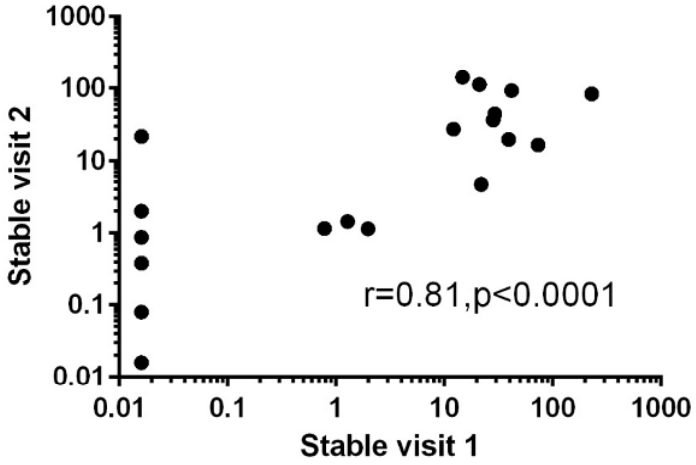

Figure E3. Correlation between 2 stable NE measurements while clinically stable before and after an exacerbation of bronchiectasis.

Changes in neutrophil elastase at exacerbation and recovery

As described in the main text, we observed increases in elastase between stability and the onset of exacerbations (p=0.002) and between exacerbation onset and recovery (p<0.0001). The median values are shown in figure E4.

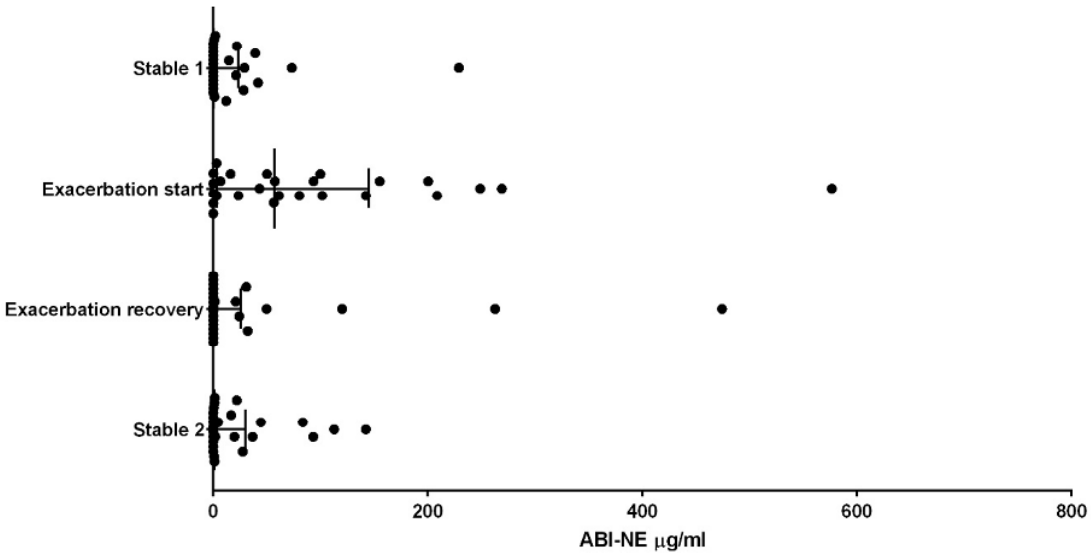

Figure E4. Changes in ABI-NE at exacerbation, recovery and longitudinal follow-up. Median with interquartile range is shown. Data are median with interquartile range.