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A point of care neutrophil elastase activity assay identifies bronchiectasis severity, airway infection and risk of exacerbation

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Introduction: Neutrophil elastase (NE) activity in sputum can identify patients at high risk of airway infection and exacerbations in bronchiectasis. Application of this biomarker in clinical practice is limited because no point of care test is available. We tested whether a novel semi-quantitative lateral flow device (Neutrophil elastase airway test stik–NEATstik®) can stratify bronchiectasis patients according to severity, airway infection and exacerbation risk.

Methods: Sputum samples from 124 patients with stable bronchiectasis enrolled in the UK and Spain were tested using the NEATstik®, which scores NE concentration from 0 (<8μg/ml elastase activity) to 10 (maximum detectable NE activity). High NE activity was regarded as a NEATstik® grade>6. Severity of disease, airway infection from sputum culture and exacerbations over the 12-months were recorded. An independent validation was conducted in 50 patients from Milan, Italy.

Measurements and Main Results: Patients had a median age of 69 years and FEV_1 69%. High NE activity was associated with worse bronchiectasis severity using the bronchiectasis severity index (p=0.0007) and FEV_1 (p=0.02). A high NEATstik® grade was associated with a significant increase in exacerbation frequency, incident rate ratio 2.75 95%CI 1.63-4.64, p<0.001. The median time to next exacerbation for patients with a NEATstik® grade >6 was 103 days compared to 278 days. The hazard ratio was 2.59 95% CI 1.71-3.94,p<0.001. Results were confirmed in the independent validation cohort.

Conclusions: A novel lateral flow device provides assessment of NE activity from sputum in minutes and identifies patients at increasing risk of airway infection and future exacerbations.

Introduction

Bronchiectasis is a complex clinical syndrome with a wide spectrum of underlying causes.[1] The outcome of bronchiectasis can be unpredictable.[2] Although there are prognostic scores to identify patients at higher risk of mortality, these have limitations including the inclusion of fixed variables such as age.[2-4] Airway infection is one of the most important "treatable traits" in bronchiectasis because chronic infection with *Pseudomonas aeruginosa* is linked to a significant increased risk of future mortality, hospital admissions and worse quality of life.[5-7] Infection with other pathogens also provokes airway neutrophilic inflammation and a higher risk of future exacerbations particularly if bacterial loads are high.[8] Alongside airway infection, exacerbations are critical events in bronchiectasis as they are associated with a significant decrement in quality of life and contribute to disease progression.[9,10]

For this reason the majority of therapies for bronchiectasis recommended by the European Respiratory Society guidelines either target the suppression of airway infection, such aslong term macrolides or inhaled antibiotics, or aim to reduce exacerbation frequency.[11,12]

A biomarker that could identify patients in real time that are at risk of future exacerbations or have active airway infection would help to identify patients in need for intensified treatment.

Neutrophil elastase (NE) activity is a leading candidate for a bronchiectasis biomarker.[13] The relationship between sputum NE activity and airway infection has been known for more than two decades and studies demonstrate that NE may directly impact on bronchiectasis disease progression through effects of ciliated epithelium, mucus production, emphysema development and inactivation of the immune response.[18-20] A systematic review of clinical studies in bronchiectasis identified 31 studies and found higher NE activity in sputum was associated with sputum colour, *P. aeruginosa* infection, airway bacterial load, disease severity, FEV_1, symptoms and long term risk of exacerbations and lung function decline.[20]

NE has therefore been tested and validated as a biomarker across multiple studies.[13, 20-22] It has not, however, been implemented into clinical practice in part due to the difficulty of standardizing sputum processing and performing ELISA or chromogenic assays in clinical laboratories. A point of care lateral flow device that could evaluate NE in minutes would overcome these limitations and may help to stratify patients in terms of disease severity and future risk.

In this study we tested the predictive value of a commercially available point of care NE assay (NEATstik®) to predict clinically relevant outcomes in patients with bronchiectasis.

Methods

Patients were enrolled while in a stable phase of their disease from specialist bronchiectasis clinics in Ninewells Hospital, UK and Hospital Sant Pau, Spain from 2016-2017. The inclusion criteria were: High resolution computed tomography confirmed bronchiectasis, the clinical syndrome of bronchiectasis defined by cough, sputum production and/or recurrent respiratory tract infections, and the ability to produce a sputum sample spontaneously at the study visit. Exclusion criteria were : known cystic fibrosis, traction bronchiectasis due to interstitial lung disease, age <18 years and inability to give informed consent to participate. The study was approved by the local research ethics committees.

Patients and clinical outcomes

Following informed consent we collected information about demographics, clinical characteristics, disease burden, previous microbiology and current treatments. Sputum samples were sent for bacterial culture and CT scans were scored using the modified Reiff scale.[2] Patients underwent spirometry according to ATS/ERS guidelines. Quality of life was evaluated using the Quality of life bronchiectasis respiratory symptoms score. The bronchiectasis severity index was calculated as previously described.[2] Data were collated in the EMBARC case report form including sputum colour measured using the Murray colour chart.[23] Chronic infection was defined as isolation of the same pathogen on two or more occasions at least 3 months apart while clinically stable in 1 year.

Sputum processing and Neutrophil elastase measurement

Neutrophil elastase was measured by two methods, a validated activity based immunoassay and the point of care "NEATstik®" test. For immunoassay, spontaneously expectorated samples were diluted with 8x volume phosphate buffered saline and centrifuged at 3000g for 15 minutes and the supernatant carefully removed as previous described.[13] DTT was not used. Samples were diluted and NE activity measured according to the manufacturers instructions. For the NEATstik® method, spontaneously expectorated sputum samples were diluted immediately 10x in buffer provided in the kit and inverted 10 times. The diluted sample was then added to the NEATstik® lateral flow device. The test consists of a control line and a test line. According to the manufacturers instructions the intensity of the test line corresponds to the neutrophil elastase concentration on a 10-point scale (figure 1) and a positive test corresponds to an approximate concentration of 8µg/ml. A video showing the testing procedure is included in the online supplement.

A subset of patients were asked to contact the study site when they experienced an exacerbation and to provide a spontaneous sputum sample. In this convenience sample, patients were reviewed by a clinician and exacerbation defined according to the British Thoracic Society definition.[24] Bacterial exacerbations were defined by the presence of a cultured pathogen and viral exacerbations defined by the presence of a positive PCR.

Validation

The reliability and reproducibility of the NEATstik® scoring system was tested. Each processed sputum sample was scored by two blinded independent observers. Observer agreement was evaluated using Bland-Altman plots, linear regression and the area under the receiver operator characteristic curve. Image analysis was conducted to provide a third validation and proof of concept that digital validation of photographs taken on a mobile phone camera could be used for future development of an app or similar assessment device. A subset of fifty images were photographed, converted to an 8-bit greyscale image and adjusted to obtain a consistent background and control line intensity. Mean pixel intensity of the NEATstik test band was quantified in FIJI (ImageJ, opensource software, NIH).

Agreement between the NEATstik® assay and the immunoassay was also tested.

Validation cohort

An independent validation study was performed at a specialist bronchiectasis clinic, Policlinico Hospital, Italy. The NEATstik® assay was performed on fresh sputum samples at point of care in 50 patients, 25 with chronic bacterial infection and 25 without chronic infection. Clinical data was collected but no follow-up data was collected in this cohort. NEATstik® results were verified by two observers.

Statistical analysis

Analysis was performed double blind, with staff performing the scoring of the NEATstik® grade blinded to patient characteristics and the staff who obtained sputum samples and collated clinical data blinded to the results of the NEATstik® test until the end of the study. For correlating NE activity with clinical parameters and long term outcomes two cut-offs were used. First, a comparison between samples testing positive (any grade 1-10) vs negative, and second a comparison between the highest NEATstik® grades (>6 vs less than or equal to 6) selected a-priori based on the correlation with the immunoassay levels of NE.

Analysis was performed using SPSS 22.0. Statistical differences among continuous variables were analysed using Student t-test and ANOVA or the appropriate non-parametrical test. Frequency of exacerbations and hospital admissions during the study were analysed using a negative binomial model with follow-up time as an offset. Time to first exacerbation was evaluated using Kaplain-Meier survival analysis. We defined statistical significance as a two-tailed $p<0.05$.

Results

Patient characteristics

124 patients were included in the study. The patient characteristics summarised in table 1 were consistent with previously reported characteristics of European bronchiectasis populations,

Demographics		
Patient characteristics		**Median (interquartile range) or N (%)**
Median (IQR) age, years		**69 (62-78)**
Female, n (%)		**71 (57.3%)**
Median (IQR) BMI,		**25.6 (22.4-29.8)**
Ex smokers, n (%)		**50 (40.3%)**
Current smokers, n (%)		**9 (7.3%)**
Aetiology		
Idiopathic, n (%)		**64 (51.6%)**
Post-infective, n (%)		**11 (8.9%)**
COPD, n (%)		**9 (7.3%)**
Post-TB, n (%)		**7 (5.6%)**
Rheumatoid arthritis, n (%)		**7 (5.6%)**
Inflammatory bowel disease, n (%)		**6 (4.8%)**
ABPA, n (%)		**3 (2.4%)**
Asthma, n (%)		**3 (2.4%)**
Immunodeficiency, n (%)		**3 (2.4%)**
Others, n (%)		**11 (8.9%)**
Disease severity		
Median (IQR) BSI score,		**7 (5-11)**
BSI score Risk Class, n (%)	**Mild**	**31 (25%)**
	Moderate	**44 (35.5%)**
	Severe	**49 (39.5%)**
Clinical status		
Sputum volume ml/day		**15 (5-30)**
MRC, median (IQR)		**2 (1-2)**
Median (IQR) exacerbations in the previous year		**2 (1-4)**

at least one hospitalization in the previous year, n (%)	**37 (29.8%)**
Functional Status	
Median (IQR) FEV_1 % predicted	**67.9 (50.9-81.5)**
Microbiology	
***P. aeruginosa* ichronic infection n (%)**	**35 (28.2%)**
Negative sputum culture n (%)	**62 (50.0%)**
Treatment	
Long term macrolide treatment, n (%)	**51 (41.1%)**
Inhaled antibiotic treatment, n (%)	**18 (14.5%)**
Quality of life	
Quality of life bronchiectasis questionnaire Respiratory Symptom Score	**59.3 (46.8-77.8)**

Table 1. Characteristics of the study population. The "other aetiologies" included alpha-1 antitrypsin deficiency, obstruction, primary ciliary dyskinesia, tracheobronchomegaly. Abbreviations: ABPA= allergic bronchopulmonary aspergillosis, BSI= bronchiectasis severity index, COPD= chronic obstructive pulmonary disease, FEV1= forced expiratory volume in 1 second, IQR= interquartile range, MRC= Medical Research Council, TB= tuberculosis

Accuracy and reproducibility of NE measurement

There was a clear and statistically significant correlation between neutrophil elastase activity measured using the immunoassay and the NEATstik® result (Spearman correlation $r=0.73, p<0.001$).

The two blinded observers showed a high level of agreement ($r=0.93, p<0.001$). There was a strong agreement between both observers and quantification of the test line by image analysis of digital photographs ($n=50$, observer1 $r=0.81$, $p<0.001$ and observer2 $r=0.91$, $p<0.001$). 21 sputum samples had no detectable NE activity (grade 0). There was agreement on 20/21 (95.2%) negative samples between observers (one awarded as grade of 1 for a single sample that the original observer rated as negative).

Based on the low number of negative results, we set a cut-off value of greater than 6 as a positive or "high risk" test. The Kappa statistic for agreement using this cut-off was 0.82 (0.73-0.91). The Bland-Altman plot is shown in figure S1. We conclude that scoring can be performed with a high degree of reproducibility.

Relationship between point of care NE assay and cross sectional severity of disease

Compared to patients without chronic infection, patients with *P. aeruginosa* had significantly higher NE activity (figure 2A) as did patients with other chronic infections ($p=0.004$). Of those without detectable NE activity, only 1 had chronic infection. The sensitivity and specificity for detection of chronic infection were 71.9% and 55.1% for a NEATstik® grade >6, and 98.3% and 31.3% for positive vs negative NEATstik® results. The corresponding sensitivity and specificity for detecting *P. aeruginosa* infection were 100% and 23.6%.

Figure 2B shows the relationship between NE and the BSI. Patients with more severe bronchiectasis had higher NE activity ($p<0.001$).The linear correlation between NE grade and BSI was $r=0.31$ (0.14-0.47,$p<0.001$). NE grade was also correlated with MRC dyspnoea score ($r=0.25$ 95% CI 0.07-0.42,$p=0.008$), FEV_1 ($r=-0.33$ 95% CI -0.49 to -0.16,$p<0.001$) and the quality of life bronchiectasis questionnaire respiratory symptom score ($r=-0.37$ 95% CI -0.56 to -0.15,$p=0.002$). Correlations, including those with bacterial infection, BSI and FEV_1 were all consistent when evaluated individually in both the Spanish and UK cohorts.

Classifying NEATstik® results as simply positive and negative also showed relationships with clinical parameters with positive tests being associated with a higher BSI score, lower FEV_1 and the presence of airway infection (figure E2).

Predictive value for future exacerbations

A high NEATstik® grade (>6) was predictive of future exacerbations. The median time to next exacerbation for patients with a NEATstik® grade >6 was 103 days compared to 278 days for those with lower NEATstik® grades. The Kaplain-meier survival curve is shown in figure 3A. The hazard ratio was 2.59 95% CI 1.71-3.94,p<0.001. Comparing positive vs negative there was similarly a statistically significant difference in time to the next exacerbation event. The median time was 135 days for patients with a positive test vs 320 days for patients with a negative test. The hazard ratio was 2.15 95% CI 1.32-3.51,p=0.01.

A high NEATstik® level was associated with a significant increase in exacerbation frequency during follow-up with an incident rate ratio of 2.75 95%CI 1.63-4.64,p<0.001 from the negative binomial model. There was consistency between the UK and Spain cohorts and in terms of prediction of exacerbations (supp Figure E3).

Severe exacerbations requiring hospitalization were infrequent during this study occurring in 20 patients (mean 0.29 per year, standard deviation 0.75). 19/20 patients requiring hospitalization had an elevated NEATstik® grade >6. The single patient with a level <6 had a negative NEATstik®. The calculated risk ratio for a NEATstik® grade >6 is 12.8 95% CI 1.77-91.9,p<0.001. The equivalent for positive vs negative NEATstik® results was 3.87 95%CI 0.55-27.4,p=0.2. It should be noted that 12/20 patients requiring hospitalization had chronic *P. aeruginosa* infection which we had already observed to be associated with high NE activity in sputum.

NE activity during exacerbations

42 patients attended for visits during protocol defined exacerbations. Details of sputum culture results at exacerbation are shown in the supplementary information. As previously observed with NE ELISA[17], changes in NE at exacerbation were heterogeneous and NEATstik® was not useful in differentiating stable state from exacerbation.

Bacterial exacerbations had higher mean NEATstik® grades as shown in figure 4. Comparisons with both viral and negative exacerbations were statistically significant (p=0.005 and p=0.001 respectively). The small numbers did not allow a meaningful comparison between viral and “negative” exacerbations.

Independent validation

The characteristics of the validation cohort are shown in Table E2 online. The relationship between NEATstik® grade and chronic infection was confirmed in this cohort (figure 5A and 5B). There was a strong agreement between two independent observers for NEATstik® grade, with the correlation for two physicians, r=0.91 (0.84-0.95,p<0.001) and for scoring performed by a nurse r=0.91 95% CI 0.84-0.95,p<0.001 and by a physiotherapist r=0.92 95% CI 0.86-0.95,p<0.001 showing similar reproducibility. NEATstik® result was not significantly correlated with the sputum colour chart (r=0.18,p=0.2) but was correlated with sputum volume r=0.39 95% CI 0.10-0.62,p=0.01. Sputum colour was not associated with BSI (p=0.06) or chronic infection (p=0.1).

Discussion

To the best of the authors knowledge, this is the first description of a rapid point of care test that can identify the presence of airway infection and the risk of future exacerbations in patients with bronchiectasis. The test is semi-quantitative and shows a good correlation with NE activity measured by immunoassay. NE activity is an established biomarker of bronchiectasis as well as being associated with risk of exacerbations, airway infection status and lung function decline in other conditions such as cystic fibrosis.[20] Implementation of sputum biomarkers into clinical practice are limited by the requirement to process sputum via centrifugation and the design of traditional assays for NE which a typically based on the cleavage of chromogenic substrates. The NEATstik® method therefore has a number of advantages over existing methods of NE quantification. The procedure requires only a sample container, a weighing balance and the NEATstik® device and a result can be obtained within minutes. It is therefore feasible to perform this test in a clinic setting or for a patient to perform the test at home.

NE is released from azurophilic granules of neutrophils during degranulation or neutrophil extracellular trap formation.[25] The strongest drivers of neutrophil recruitment to the airway and subsequent degranulation are bacterial infection.[8] Several studies have shown that patients with *P. aeruginosa* have high levels of NE activity.[8,13,21,22] Previous work also shows a strong relationship between NE activity and bacterial load, but for pragmatic reasons we did not perform quantitative bacteriology in this study. Patients with more severe bronchiectasis had higher NE activity in this study as previously reported.[13] The BSI is a composite score that includes age, BMI, MRC dyspnoea score, radiological bronchiectasis, exacerbations, hospitalisations, FEV1 and chronic infection. We show here that the relationship between NE activity and severity is driven by worse symptoms of breathlessness, more exacerbations, worse lung function and more chronic infection in patients with higher levels of NE.

We identified very few samples (21/124 in stable patients) that were truly negative using the NEATstik® assay. For this reason the semi-quantitative scoring may be most useful in clinical practice since a positive/negative approach may be too sensitive and insufficiently specific. On the other hand, the 100% sensitivity we observed for *P. aeruginosa* infection suggests that, if validated in further independent cohorts, a negative test could be used to effectively exclude the presence of *P. aeruginosa* infection without requiring culture. We found that the semi-quantitative grading could be performed reliably and reproducibly between independent observers.

Exacerbations are key drivers of disease progression in bronchiectasis. Elevated NE predicted a shorter time to the next exacerbation and a higher frequency of exacerbations during follow-up. The magnitude of this effect with the NEATstik® method was similar to that previously observed with an immunoassay in an independent cohort. The prediction of exacerbations to guide preventative therapy is a key unmet need in the management of bronchiectasis patients. The European Respiratory Society guidelines recommend long term macrolides, long term inhaled antibiotics and some mucoactive therapies to patients at risk of future exacerbations but at present the only method available to predict future exacerbations is the history of previous exacerbations.[9] While these are strongly correlated, recent experience suggests it is not always reliable. In the RESPIRE trials of inhaled dry powder ciprofloxacin the inclusion criteria required at least 2 exacerbations in the previous year.[27-29] The subsequent rates of exacerbation of 1 per year to 0.6 per year in the placebo groups of these trials suggest a failure to enrich for patients at high risk of events. A point of care test that can identify patients at risk would therefore be useful in clinical practice to guide therapy and to enrich clinical trials.

We performed a pilot study of 42 exacerbations to investigate if different exacerbation types had different levels of NE. We found a clear association between elevated NE positive bacterial culture at exacerbation. It is important to note that negative NEATstik® tests were observed at exacerbation confirming that NE cannot be used to diagnose exacerbation since there appear to be phenotypes of exacerbation which are not associated with elevated neutrophilic inflammation.[13] Nevertheless our initial observation would suggest the future potential to guide antibiotic treatment of exacerbations based on the NEATstik® result.

Our study has important limitations. The sample size was sufficient to demonstrate statistically significant differences between the groups but given the heterogeneity of bronchiectasis it would be important to investigate the NEATstik® assay in a larger cohort of patients. Our study was limited to 12 months follow-up and there were relatively few severe exacerbations on which to base any strong conclusions regarding this endpoint. As the participating hospitals are tertiary referral centres the study included a high proportion of patients with severe bronchiectasis and therefore care should be taken if extrapolating results to a milder population. Our independent validation cohort included only 50 patients and was designed to validate the association with airway infection and was not powered to test the association with exacerbations. Further large studies are therefore still needed. An important limitation of our respiratory infection data is the reliance on traditional culture methods. Studies are increasingly using more sensitive methods such as quantitative PCR or sequencing of the airway microbiome to define airway infection.[30] These methods are not yet implemented into clinical practice and so we regard culture as an appropriate gold standard for our analysis, albeit one with important limitations. Despite these limitations our study has many strengths including the extensive validation of the assay, the multicentre design with consistent results between Spain, Italy and the UK and the comprehensive clinical data recorded.

In summary, a simple point of care semi-quantitative NE assay can identify bronchiectasis patients with airway infection and patients at high risk of exacerbations over the subsequent 12 months. Future studies should evaluate whether implementation of such an assay in clinical practice or clinical trials can be used to improve risk stratification or therapeutic decision making.

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Figure legends

Figure 1. NEATstik® scoring system. A value of 0-10 is awarded based on the intensity of the band.

Exacerbation study

Figure 2. The relationship between the NEATstik® grade, airway infection and severity of disease. A= airway infection, B= Bronchiectasis severity index, C= FEV1 groups. Data are shown as mean with standard error of the mean. Coloured error bars are to improve visualisation rather than having any significance.

Figure 3. Time to first exacerbation according to baseline NEATstik® NE result. High refers to NEATstik® grade >6. Positive refers to any grade >0.

Figure 4. Neutrophil elastase NEATstik® results at exacerbation in 42 patients. Bacterial exacerbations (n=26), viral exacerbations (n=8) and exacerbations where both sputum culture and viral PCR were negative. Errors bars shown mean with standard error of the mean.

Figure 5. Chronic infection status and NEATstik® grade. A) All chronic infection including *P. aeruginosa* and other pathogens B) separating *P. aeruginosa* and other pathogens**.**