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Original research

Clinical, molecular and microbial characterisation of the eosinophilic endotype of bronchiectasis: data from the EMBARC-BRIDGE study

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ABSTRACT

Objectives Eosinophilic bronchiectasis is defined by a blood eosinophil count (BEC) ≥300 cells/µL, but blood eosinophils imperfectly reflect airway eosinophilic inflammation. Here, we investigated the relationship between eosinophilic airway inflammation, blood eosinophils and clinical severity in bronchiectasis and explored the phenotype associated with eosinophilic bronchiectasis.

Methods Sputum from 180 patients with stable CT-confirmed bronchiectasis was utilised to investigate airway levels of eosinophil proteins (eosinophil peroxidase (EPX), eosinophil derived-neurotoxin (EDN), eosinophil cationic protein (ECP), major basic protein (MBP) and Galectin-10 (Gal-10)) using a novel stable isotope dilution liquid chromatography-tandem mass spectrometry (LC-MS/MS) assay. To profile eosinophilic bronchiectasis, a nested analysis of patients with BEC <150 cells/µL (n=52) and ≥300 cells/µL (n=49) was conducted.

Results Sputum concentrations of Gal-10, ECP and EDN were weakly but significantly associated with radiological severity, FEV_1 and sputum culture positivity for *Pseudomonas aeruginosa*. Airway eosinophil protein concentrations did not associate with exacerbation frequency. Total eosinophil protein concentration moderately correlated with BECs (r=0.33 95% CI 0.14 to 0.49, p=0.0007). Nested analysis revealed increased sputum PCR-positivity for *P. aeruginosa* (26.7% vs 7.7%, p=0.033) and an increased frequency of patients showing signs of *Aspergillus* sensitisation (defined as *Aspergillus*-specific IgE titres >0.35 kUA/L, 24.5% vs 3.8%) in eosinophilic bronchiectasis. Sputum inflammatory biomarkers and clinical parameters did not differ between groups.

Conclusions LC-MS/MS can detect eosinophilic inflammation within bronchiectasis sputum. Weak associations between elevated airway eosinophil proteins, bronchiectasis severity and *P. aeruginosa* infection were observed. Direct measurement of eosinophilic airway inflammation provides additional information in addition to BECs. Eosinophilic bronchiectasis associated with *P. aeruginosa* infection and *Aspergillus* sensitisation.

WHAT IS ALREADY KNOWN ON THIS TOPIC

⇒ Eosinophilic bronchiectasis is a newly described endotype of disease—defined by a blood eosinophil count (BEC) ≥300 cells/µL—representing ~20% of all bronchiectasis cases. This disease endotype remains poorly understood. BECs are recognised as an imperfect surrogate of eosinophilic airway inflammation, and the relationship between BECs, airway eosinophilic inflammation and bronchiectasis severity is unclear.

WHAT THIS STUDY ADDS

⇒ We developed and utilised a novel mass spectrometry-based proteomic assay for the parallel detection and quantification of airway eosinophil proteins in sputum from patients with bronchiectasis. We identified a moderate correlation between airway eosinophil proteins and BECs. Elevated airway eosinophil protein concentrations were also weakly associated with worsened radiological severity, reduced lung function and airway *Pseudomonas aeruginosa* infection. In a nested analysis of patients with BECs <150 cells/µL and ≥300 cells/µL, elevated BECs were associated with sputum PCR-positivity for *P. aeruginosa* and *Aspergillus* sensitisation.

INTRODUCTION

Bronchiectasis is a chronic lung disease driven by a complex interconnected cycle of airway inflammation, chronic infection and defective mucociliary clearance.[1] Historically, inflammation in bronchiectasis was thought to be predominantly neutrophilic; however, an eosinophilic endotype of bronchiectasis, defined by a blood eosinophil count (BEC) ≥300 cells/µL despite the absence of eosinophil-associated conditions, asthma and/or allergic bronchopulmonary aspergillosis (ABPA), accounting for ~20% of cases has been described.[2 3]

HOW THIS STUDY MIGHT AFFECT RESEARCH, PRACTICE OR POLICY

⇒ This study suggests a weak association between eosinophilic airway inflammation and clinical severity in bronchiectasis, including infection with P. aeruginosa. In addition, this study highlights that direct measurement of eosinophilic airway inflammation in sputum may complement or enhance information obtained from the use of BECs. Taken together, we provide evidence that eosinophilic inflammation is frequently present in bronchiectasis and may therefore be an important treatment target, with widely available therapeutics such as inhaled corticosteroids or biologic drugs.

Eosinophils—innate granulocytic cells with a primary role in type 2 (T2)-mediated immunity—have a well-recognised and longstanding role in lung pathology and disease severity in asthma. In bronchiectasis, the influence of eosinophilic inflammation on disease severity and activity is less clear. Elevated BECs seemingly represent a risk factor for bronchiectasis exacerbation, with a suggested link between eosinophilic disease and bronchiectasis severity having been reported.[2–4] However, studies characterising this disease endotype are limited and report conflicting findings.[5] In turn, while BECs represent a surrogate marker for eosinophilic airway inflammation in bronchiectasis,[3] they appear imperfect, and consequently, the clinical significance of eosinophilic airway inflammation in bronchiectasis is unclear. The measurement of eosinophil granule proteins in sputum is infrequent, partly due to an incompatibility between commercial assays and sputum and/or an inability of such assays to detect low protein concentrations. Sputum cell counts are also not universally available for those with bronchiectasis; therefore, correlations between direct measures of eosinophilic airway inflammation and bronchiectasis disease severity or treatment response are lacking.

Pseudomonas aeruginosa, an opportunistic pathogen associated with bronchiectasis severity,[6] reportedly shows increased abundance in the airways of those with bronchiectasis with elevated BECs ≥300 cells/µL compared with those with low (<100 cells/µL) and normal (100–299 cells/µL) BECs using 16S rRNA sequencing.[3] *P. aeruginosa* has also been reported to associate with robust T2 responses in cystic fibrosis (CF),[7 8] despite CF also being a predominantly neutrophil-driven disease akin to bronchiectasis, further suggesting a potential role for *P. aeruginosa* in eosinophilic bronchiectasis.

In turn, ABPA is a known cause and consequence of bronchiectasis, with eosinophilia being a key diagnostic feature of ABPA. *Aspergillus* sensitisation, where individuals show hypersensitivity towards *Aspergillus* species in the form of *Aspergillus*-specific IgE and total IgE, has been associated with worsened lung function and poorer clinical outcomes in bronchiectasis.[9] As eosinophilia in bronchiectasis is present in individuals without ABPA, this begs the question of whether similar hypersensitivity responses towards *Aspergillus* species drive eosinophilic bronchiectasis, thus representing a clinical entity on the spectrum of allergic *Aspergillus* disease.

Eosinophilia represents a treatable trait in asthma and chronic obstructive pulmonary disease (COPD), and BECs (typically ≥300 cells/µL) are currently used in these conditions to guide treatment with numerous T2-targeting biologic therapies. Small case series highlight a treatment benefit of anti-IL-5 biologics in those with severe asthma with concomitant bronchiectasis.[10 11] Given the success of eosinophil-targeting therapies in these contexts, BECs are being used to guide off-label use of anti-eosinophil therapies, such as inhaled corticosteroids (ICS) and anti-interleukin-5 (anti-IL-5)/IL-5 receptor biologics, in those with bronchiectasis with elevated BECs, with reports of a treatment benefit.[12–14] However, as the clinical significance of eosinophils in bronchiectasis remains poorly understood, no large trials investigating such therapies in those with eosinophilic bronchiectasis have been conducted.

This study aimed to (1) investigate eosinophilic airway inflammation in bronchiectasis through the measurement of sputum eosinophil proteins by mass spectrometry; (2) explore the relationship between eosinophilic airway inflammation, BEC and bronchiectasis disease severity; and (3) characterise the eosinophilic endotype of bronchiectasis by examining the clinical, microbial and inflammatory features of those with eosinophilic bronchiectasis.

METHODS

Study cohort

This was a single centre study of patients with CT-confirmed bronchiectasis conducted at Ninewells Hospital, Dundee, UK. Patients met the following inclusion criteria: (1) clinical history compatible with bronchiectasis including cough, sputum production and/or recurrent chest infections; (2) primary diagnosis of bronchiectasis established by a respiratory physician; and (3) were clinically stable, indicated by a lack of antibiotic and/or oral corticosteroid treatment for pulmonary exacerbation within 4 weeks prior to enrolment. Exclusion criteria included an inability to provide informed consent, age <18 years, active tuberculosis or non-tuberculosis mycobacterium at enrolment and bronchiectasis resulting from CF. Patients were also selected based on their BEC on initial recruitment (with an enrichment for those with a BEC ≥300 cells/µL or BEC ≤150 cells/µL). All patients gave informed consent to participate, and the study was approved by the local research ethics committee.

To characterise eosinophilic bronchiectasis, from the above cohort, a subset of patients without a physician's diagnosis of asthma and/or active ABPA (according to diagnostic guidelines[15]) with a baseline BEC ≥300 cells/µL (representing eosinophilic disease based on current bronchiectasis literature)[2–4 12 14] or <150 cells/µL (representing non-eosinophilic disease based on thresholds widely used in COPD (reviewed in[16] as no validated threshold for identifying non-eosinophilic disease in bronchiectasis has been defined)) were selected. Their clinical, microbial and inflammatory features were compared using the methods described below. While these patient groups were not formally matched, efforts were made to enrol patients with a similar age and sex distribution.

A graphical overview of the patient cohorts used is shown in figure 1.

Data collection

Comprehensive clinical information including demographics (age, sex, body mass index, etc), medical history (ie, symptoms, exacerbations in the previous year, concomitant respiratory and non-respiratory medications, comorbidities, etc), smoking history, radiology, spirometry, microbiology (based on historical sputum cultures obtained from samples taken as part of routine clinical care) and aetiological testing were collected on enrolment as previously described.[17 18] The Bronchiectasis Severity Index (BSI)[19] was used to establish disease severity. Pulmonary exacerbation was defined as 'the use of antibiotics for acute worsening of respiratory symptoms' based on the consensus

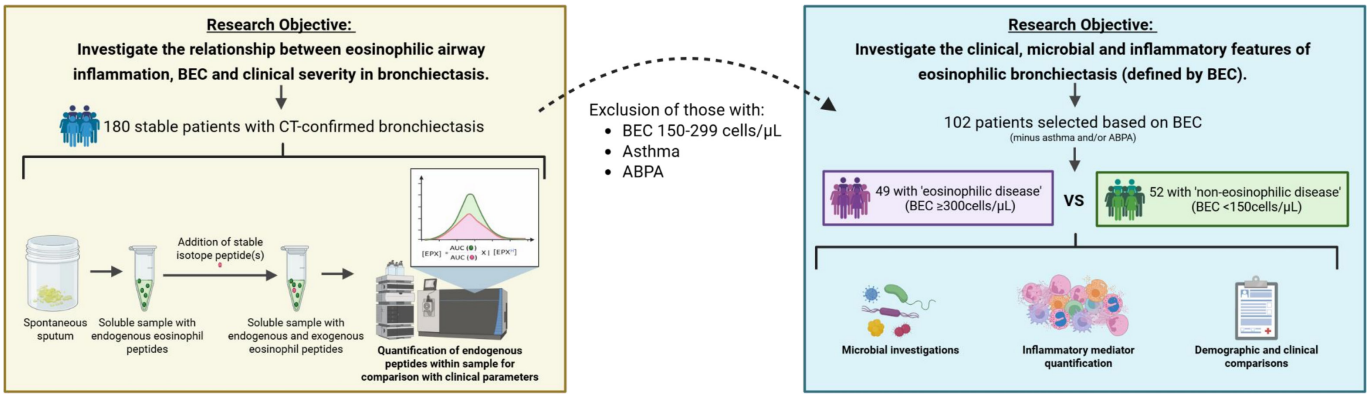

Figure 1 Graphical overview of research objectives, patient cohorts and methods used within this study. ABPA, allergic bronchopulmonary aspergillosis; BEC, blood eosinophil count.

definition of pulmonary exacerbations for clinical research in adult patients with bronchiectasis plus the requirement for antibiotic treatment.[20]

Spontaneously expectorated sputum and blood were collected at baseline.

Measurement of airway eosinophil proteins

Eosinophil proteins in sputum supernatant were quantified using a novel stable isotope dilution liquid chromatography-tandem mass spectrometry (LC-MS/MS)-based assay. Five heavy-labelled peptide internal standards from eosinophil peroxidase (EPX), eosinophil derived-neurotoxin (EDN), eosinophil cationic protein (ECP), major basic protein (MBP) and Galectin-10 (Gal-10) were used as references and spiked into sputum digests.

Full details are available in online supplemental table 1.

Assessment of airway microbiology

Multiplex PCR (BioFire)

The presence/absence of respiratory pathogens in whole sputum was detected by multiplex PCR using the BioFire FilmArray Pneumonia plus Panel with the FilmArray 2.0 multiplex PCR system and associated software from Biomerieux (St-Laurent, Canada) according to the manufacturer's instructions.

Aspergillus RT-qPCR

Real-time quantitative PCR (RT-qPCR) was performed to detect the presence/absence of *A. fumigatus* within patient sputum samples using modified methods based on those previously described.[21] The assay specifically detects the *A. fumigatus* ITS1 region (18S rRNA).

RT-qPCR was performed using a QuantStudio 7 Flex Real-Time PCR machine (Applied BioSystems). Samples, water and positive control were tested in triplicate, with DNA extraction negatives used as contamination controls. Samples with a Cycle Threshold value <40 were considered positive for *A. fumigatus*, as previously described in.[21]

Full details regarding microbiology assessment methods and analysis are available in online supplemental material 2.

Quantification of inflammatory mediators

Sputum supernatant and serum were used for the measurement of airway and systemic levels of an array of physiologically relevant inflammatory mediators. Airway and systemic levels of Eotaxin, Eotaxin-2, Eotaxin-3, GM-CSF, IFNγ, IL-13, IL-17A, IL-2, IL-4, IL-5, TARC, G-CSF, Fractalkine, IL-17E/IL-25, IL-22, IL-33 and TSLP, IL-3 were quantified by multiplex cytokine immunoassay (Mesoscale Diagnostics). Sputum levels of IL-8 were quantified by ELISA (R&D Systems). Active neutrophil elastase and NET complexes (by DNA-elastase assay) were quantified as previously described.[22 23]

Statistical analyses

Demographic data are presented as median with IQR unless otherwise stated. Comparisons of continuous parameters (cytokine data and/or clinical parameters) between two groups were performed using the Mann-Whitney U test. A Kruskal-Wallis test was used for comparisons of more than two groups, with a Dunn's multiple comparisons test to establish significant differences between each pair of comparisons. Where appropriate, adjustment for multiple testing was performed using the Bonferroni-Dunn method. To compare differences in categorical parameters between groups, a $\chi 2$ test was performed. For assessing the relationships between inflammatory markers, Spearman's correlation was conducted. To address potential confounding of ICS use, sensitivity analyses, whereby all individuals receiving ICS were removed, were performed where appropriate. All hypothesis tests were two-sided, and assumptions were assessed graphically. All analysis was performed using GraphPad Prism (V.9.0.2) and/or SPSS (V.29.0.1.0).

RESULTS

Study overview

A visual summary of the study is provided in figure 1.

Detection and quantification of eosinophil granule proteins within bronchiectasis patient airways and their link with disease severity and bronchiectasis outcomes

A novel assay was developed for the targeted detection and quantification of the five key cytotoxic eosinophil proteins (Gal-10, ECP, EPX, EDN and MBP) in sputum.

Airway concentrations of eosinophil proteins were quantified in sputum supernatant from 180 patients with stable bronchiectasis. Patient demographics are described in table 1. Patients were representative of a European bronchiectasis cohort, being predominantly female with a median age of 69 years. 63 patients (35.1%) had a BEC ≥300 cells/µL.

All measured eosinophil proteins were detectable within sputum supernatant, with correlation analysis revealing strong, highly significant correlations between all eosinophil proteins (online supplemental figure 1).

To assess whether eosinophilic airway inflammation associates with clinical severity, airway eosinophil protein levels were

Table 1 Cohort demographics of bronchiectasis patients included in the airway eosinophil protein quantification study

Variables	Airway eosinophil protein quantification study cohort
n	180
Demographics	
Age, years (median, IQR)	69 (62–76)
Female, n (%)	98 (54.4%)
BMI, kg/m^2 (median, IQR)	26.6 (23.5–30.4)
Smoking status, n (%)	
Never	88 (49.2%)
Ex	76 (42.4%)
Current	15 (8.4%)
BEC classification, n (%)	
≥300 cells/µL	63 (35%)
150–300 cells/µL	19 (10.6%)
≤150 cells/µL	98 (54.4%)
Clinical status (median, IQR)	
Bronchiectasis Severity Index score	6 (4-9)
Number of exacerbations in previous year	1 (0–3)
Reiff radiological severity score	3 (2-5)
Sputum volume (mL)/day	15 (5–30)
MRC dyspnoea score, n (%)	
0	39 (21.8%)
1	55 (30.7%)
2	29 (16.2%)
3	32 (17.8%)
4	24 (13.4%)
Lung function (median, IQR)	
FEV_1 (L)	1.9 (1.4–2.5)
FEV_1 % predicted	79.7 (58.6–101.6)
FVC (L)	3.2 (2.4–4.0)
FVC % predicted	100.1 (84.4–116.8)
Treatments (n %)	
Inhaled corticosteroids (ICS)	74 (41.3%)
Long-term macrolide/oral antibiotic	54 (30.2%)

Data are shown as median with IQR or n. (%).
BEC, blood eosinophil count; BMI, body mass index; FEV_1, forced expiratory volume in 1 second; FVC, forced vital capacity; ICS, inhaled corticosteroids; MRC, Medical Research Council.

compared with clinical severity parameters. Airway eosinophilic inflammation correlated with lung function impairment and extent of radiological disease, shown by weak statistically significant inverse correlations between FEV_1 and Gal-10 (Spearman r=−0.20, p=0.01, figure 2A), ECP (Spearman r=−0.27, p=0.0007, figure 2B) and EDN (Spearman r=−0.24, p=0.002, figure 2C) and weak statistically significant positive correlations between Reiff radiological severity score and Gal-10 (Spearman r=0.24, p=0.001, figure 2D), ECP (Spearman r=0.24, p=0.004, figure 2E) and EDN (Spearman r=0.23, p=0.002, figure 2F). No relationships were observed between these parameters and MBP or EPX concentrations (online supplemental figure 2).

No relationships were observed between any of the measured airway eosinophil proteins, when analysed individually or when considered as a cumulative concentration (represented as 'All Eosinophil Proteins') and overall disease severity as measured by the BSI score (data shown for 'All Eosinophil Proteins'; p=0.25, Kruskal-Wallis, figure 3A). Likewise, no relationships were observed between any of the measured airway eosinophil proteins and exacerbations (data shown for 'All Eosinophil Proteins'; p=0.21, Kruskal-Wallis, figure 3B) and/or severe exacerbations requiring hospitalisations in the previous year (data shown for 'All Eosinophil Proteins'; p=0.72, Mann-Whitney, figure 3C).

Interestingly, airway concentrations of Gal-10, ECP and EDN were significantly elevated in those sputum culture positive for *P. aeruginosa* (Gal-10 p=0.002; ECP p=0.007; EDN p=0.002, Mann-Whitney, figure 4), further supporting an association between *P. aeruginosa* and eosinophilia in bronchiectasis.

The relationship between peripheral blood eosinophilia and airway eosinophilic inflammation

Given existing literature suggesting poor reliability of BECs as a surrogate for eosinophilic airway inflammation, BECs and airway eosinophil protein concentrations were compared.

Weak positive correlations were observed between eosinophil proteins and BECs (table 2). When patients were stratified as 'eosinophilic' (BEC ≥300 cells/µL) or 'non-eosinophilic' (BEC <150 cells/µL), cumulative eosinophil protein concentration was significantly increased in the airways of those with eosinophilic disease by BEC (p=0.003, Mann-Whitney, figure 5A), although with significant overlap in protein concentrations between BEC groups. When analysed individually, only Gal-10, ECP and EPX appeared significantly elevated among those with elevated BECs (Gal-10 p=0.001; ECP p=0.02; EPX p<0.0001, Mann-Whitney, figure 5B–D).

Characterising eosinophilic bronchiectasis

Demographic and clinical features of eosinophilic bronchiectasis

We next aimed to characterise the clinical, microbial and inflammatory characteristics of eosinophilic bronchiectasis. To do so, a subset of individuals from the analyses above with high or low BECs—representing the 'extremes' of the eosinophil inflammatory spectrum—were analysed further to assess the influence of eosinophils in bronchiectasis. 102 patients were included for analysis: 49 with eosinophilic disease (BEC ≥300 cells/µL) versus 53 with non-eosinophilic disease (BEC <150 cells/µL). Eosinophilic and non-eosinophilic individuals were well balanced with regards to age and sex. Patient characteristics are shown in table 3.

Table 3 highlights a significantly increased prevalence of idiopathic disease among those with eosinophilic disease (73.5% vs 45.3%, $\chi 2$, p=0.004), as well as a higher use of ICS (57.1% vs 30.2%, Chi-square, p=0.006). No significant differences in various clinical parameters were observed between the BEC groups, including radiological severity (p=0.885, Mann-Whitney), daily sputum volume (p=0.194, Mann-Whitney) and MRC dyspnoea group classification (p=0.117, $\chi 2$). No significant differences in lung function (p=0.484, Mann-Whitney), overall BSI scores (p=0.849, Mann-Whitney) and the frequency of exacerbations (p=0.961, $\chi 2$) or severe exacerbations requiring hospitalisation (p=0.500, $\chi 2$) in the previous 12 months were observed.

With the proposed clinical benefit of ICS in those with elevated BECs,[12 14] it was hypothesised that ICS use may be confounding relationships between BEC and clinical phenotype. Sensitivity analysis, whereby patients were further stratified by ICS status

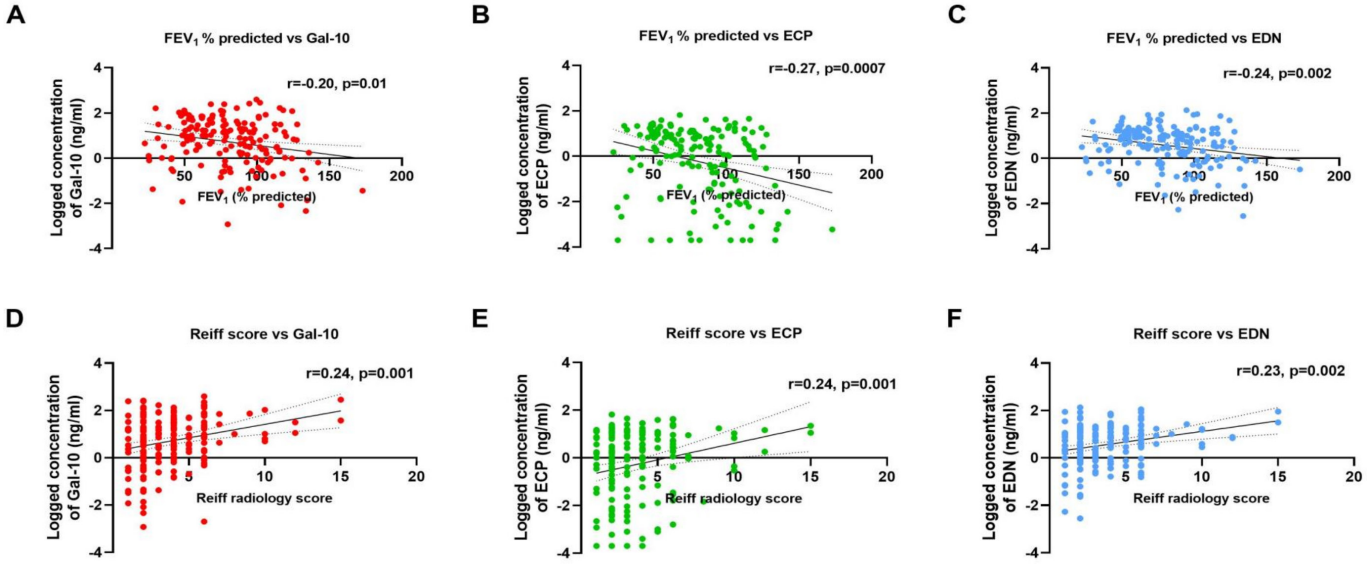

Figure 2 Eosinophil proteins Gal-10, ECP and EDN inversely correlate with lung function (A–C) and positively correlate with radiological severity (D–F). Lung function measured as FEV_1% predicted. Radiological severity measured by Reiff radiological severity score. Correlation analysis was performed using Spearman correlation using non-log transformed concentrations of eosinophil granule proteins. ECP, eosinophil cationic protein; EDN, eosinophil-derived neurotoxin; FEV_1% predicted, forced expiratory volume in 1 second % of predicted; Gal-10, Galectin-10.

and their clinical parameters were compared, revealed no differences in clinical parameters or overall severity between the BEC groups (online supplemental table 2).

Interestingly, significantly elevated total IgE and *Aspergillus*-specific IgE titres were observed among those with eosinophilic disease (*Aspergillus*-specific IgE p=0.005; total IgE p=0.033, Mann-Whitney, table 3), with a higher proportion of patients in the eosinophilic group meeting the diagnostic criteria for *Aspergillus* sensitisation (defined as *Aspergillus*-specific IgE titres >0.35 kUA/L, 24.5% vs 3.8%). No significant difference in *Aspergillus*-specific IgG was observed (p=0.625, Mann-Whitney, table 3).

Microbial features of eosinophilic bronchiectasis

To assess the microbial profiles of those with eosinophilic disease, multiplex PCR was performed for the simultaneous detection of common bacterial and viral respiratory pathogens in patient sputum for those with sputum available (n=69). Here, *P. aeruginosa* was more frequently detected in those with eosinophilic disease, an association found to be significant (26.7% vs 7.7%, p=0.033, $\chi 2$, figure 6A). Bacterial culture results appeared largely concordant with PCR results for those PCR-positive for *P. aeruginosa* (concordance rate=66.7%) (online supplemental table 3). No significant differences in other bacterial or viral pathogens were observed between the BEC groups (online supplemental table 4).

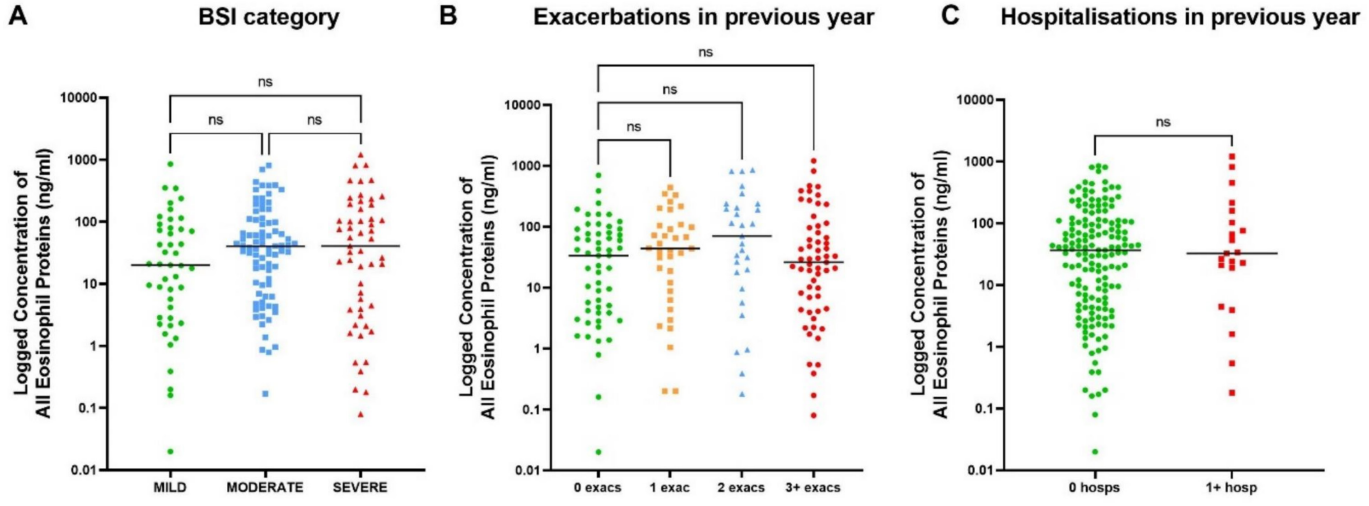

Figure 3 Airway eosinophil proteins do not associate with bronchiectasis severity index (A), exacerbation frequency within the previous year (B) or hospitalisation frequency within the previous year (C). 'All Eosinophil Proteins' represents the cumulative concentration of all five measured eosinophil proteins. Data are presented as logged values of the mean protein concentration (measured in ng/mL). Pairwise comparisons were performed using Mann-Whitney (continuous variables, two groups) or Kruskal-Wallis test (continuous variables, 3+ groups) with non-log transformed concentrations of eosinophil granule proteins. BSI, bronchiectasis severity index; exacs, exacerbations; hosps, hospitalisations; ns, not significant.

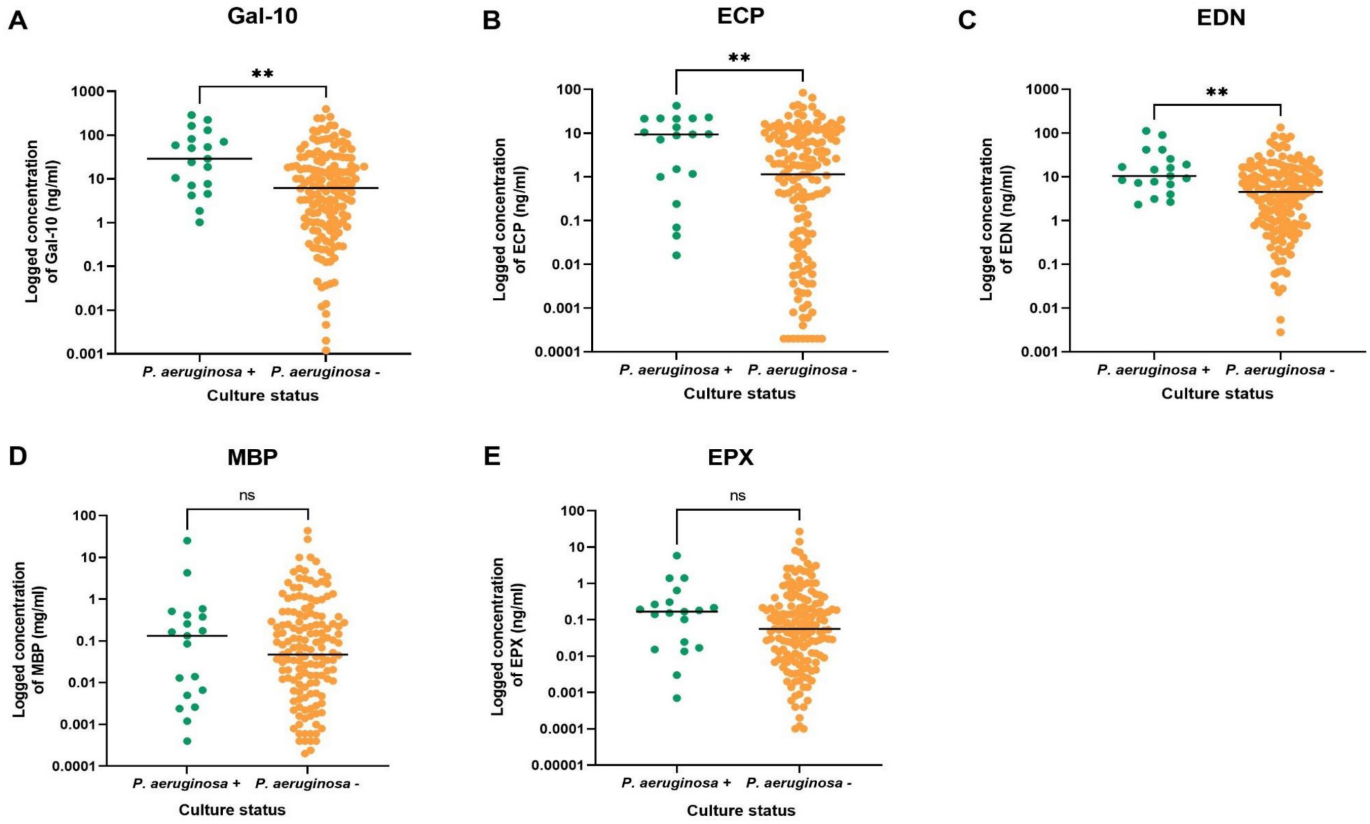

Figure 4 Eosinophil granule proteins Gal-10 (A), ECP (B) and EDN (C), but not MBP (D) or EPX (E), are elevated in those culture positive for *Pseudomonas aeruginosa*. Data are presented as logged values of the mean protein concentration (measured in ng/mL). Pairwise comparisons were performed using the Mann-Whitney test with non-log transformed concentrations of eosinophil granule proteins. ECP, eosinophil cationic protein; EDN, eosinophil-derived neurotoxin; EPX, eosinophil peroxidase; Gal-10, Galectin-10; MBP, major basic protein.

Among those with sputum DNA available (n=94), RT-qPCR revealed no significant difference in the presence of airway *Aspergillus fumigatus* between eosinophilic and non-eosinophilic disease (25.6% vs 19.6%, p=0.489, $\chi 2$, figure 6B), in line with our above data showing no difference in *Aspergillus*-specific IgG between the patient groups (table 3).

Table 2 Airway levels of eosinophil granule proteins positively correlate with blood eosinophil counts

Eosinophil granule protein vs BEC	Spearman R correlation coefficient	P value	Adj p value
All eosinophil proteins vs BEC	0.33 (0.14–0.49)	0.0007	0.004
Gal-10 only vs BEC	0.32 (0.13–0.48)	0.001	0.007
ECP only vs BEC	0.22 (0.02–0.40)	0.024	0.144
EPX only vs BEC	0.34 (0.15–0.50)	0.0005	0.003
EDN only vs BEC	0.34 (0.15–0.50)	0.0005	0.003
MBP only vs BEC	0.30 (0.11–0.47)	0.002	0.013

'All eosinophil proteins' represents the cumulative concentration of all five measured eosinophil proteins.
Correlation analysis was performed using Spearman correlation with non-log transformed concentrations of eosinophil granule proteins.
P values adjusted for multiple testing using Bonferroni-Dunn method.
Adj, adjusted; BEC, blood eosinophil count; ECP, eosinophil cationic protein; EDN, eosinophil-derived neurotoxin; EPX, eosinophil peroxidase; Gal-10, Galectin-10; MBP, major basic protein.

Inflammatory features of eosinophilic bronchiectasis

Finally, the airway and systemic inflammatory profiles of eosinophilic and non-eosinophilic bronchiectasis patients were investigated. Overall, sputum and serum inflammatory mediators were poorly correlated (online supplemental table 5). In serum, IL-5 and thymus and activation regulating chemokine (TARC) were significantly increased in those with eosinophilic disease (IL-5 p<0.0001; TARC p=0.0048, Mann-Whitney); however, airway concentrations of these mediators (IL-5 p=0.202; TARC p=0.522, Mann-Whitney) and neutrophil-associated mediators (NE p=0.248; IL-8 p=0.643, Mann-Whitney; NETs $\chi 2=2.199$, p=0.333, $\chi 2$) did not differ between the BEC groups (online supplemental figure 3). Cluster analysis revealed considerable inflammatory heterogeneity between the BEC groups (online supplemental figure 4), even in sensitivity analysis accounting for ICS use (online supplemental figure 4).

DISCUSSION

Eosinophilic bronchiectasis, defined by a BEC ≥300 cells/µL despite the absence of asthma and/or ABPA, accounts for ~20% of all bronchiectasis cases,[3] yet remains poorly understood. It is unclear whether elevated eosinophils truly contribute to disease severity and whether systemic eosinophil levels accurately reflect the airway environment. In turn, whether eosinophilic disease manifests as a distinct observable phenotype is unknown, with the cause of the observed eosinophilia in this patient group also being unclear.

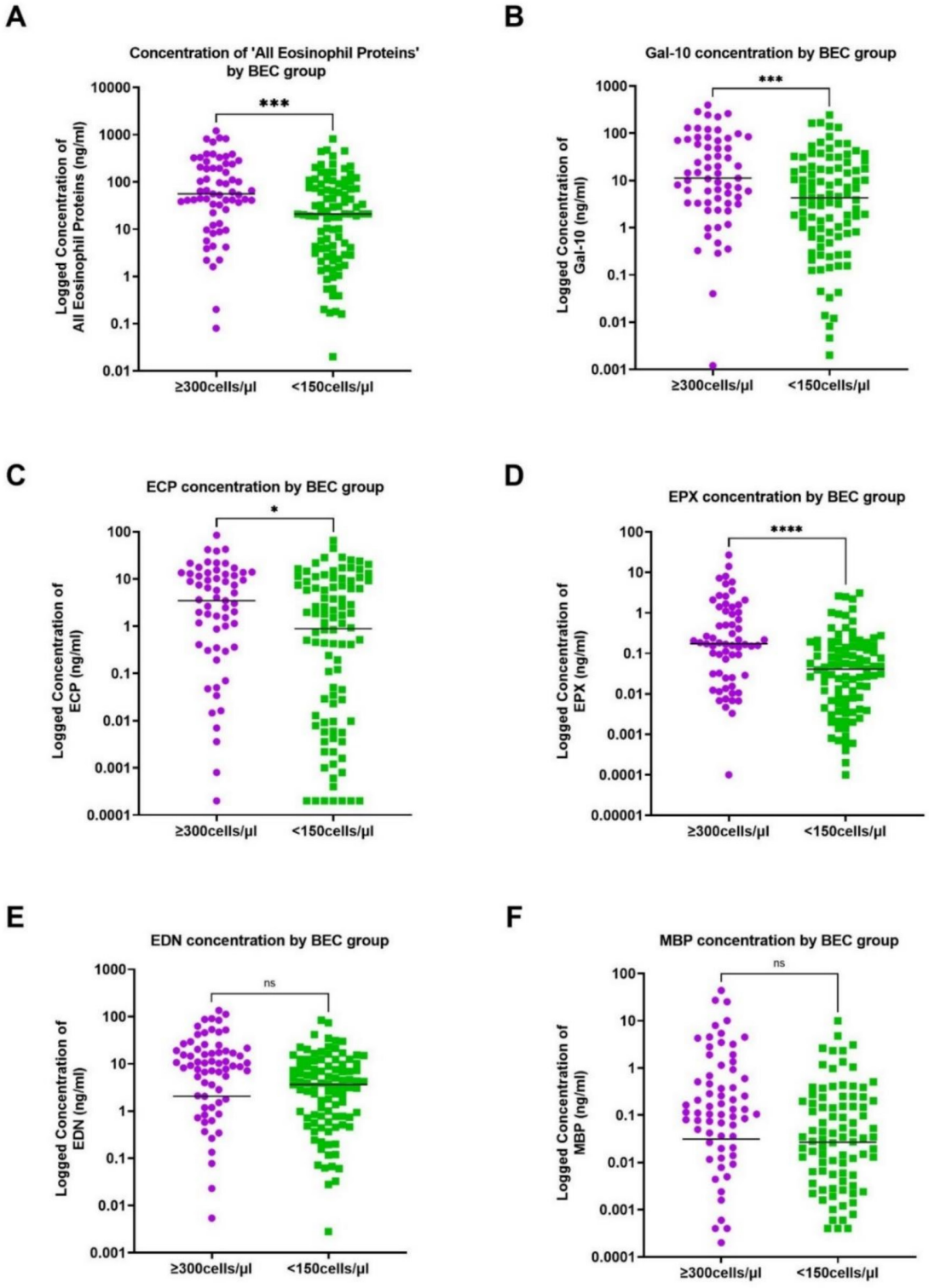

Figure 5 Eosinophil granule proteins are significantly elevated in those with BEC ≥300 cells/µl in comparison to those with BEC <150 cells/µL. 'All Proteins' represents the cumulative concentration of all five measured eosinophil proteins (A). Concentrations of individual proteins across BEC groups (B–F). Data are presented as logged values of the mean protein concentration (measured in ng/mL). Pairwise comparisons were performed using the Mann-Whitney test with non-log transformed concentrations of eosinophil granule proteins. BEC, blood eosinophil count; ECP, eosinophil cationic protein; EDN, eosinophil-derived neurotoxin; EPX, eosinophil peroxidase; Gal-10, Galectin-10; MBP, major basic protein.

This study used a novel LC-MS/MS assay designed to detect and quantify eosinophil granule proteins within patient sputum, with the aim of assessing the relationship between eosinophilic airway inflammation, BECs and bronchiectasis disease severity. Using this method, we were able to successfully detect and quantify each of the five key eosinophil proteins (ECP, EDN, EPX, MBP and Gal-10) within bronchiectasis sputum.

The observed lack of association between eosinophilic airway proteins and exacerbations or overall disease severity (as indicated by the BSI) may reflect the similar ages and exacerbation

Table 3 Detailed patient characteristics of those with eosinophilic bronchiectasis (BEC ≥300 cells/µl) and those with non-eosinophilic bronchiectasis (BEC <150 cells/µL)

Variables	Eosinophilic **(BEC ≥300 cells/µL)**	Non-eosinophilic **(BEC <150 cells/µL)**	P value
(%)	49 (48.0%)	53 (52.0%)	
Demographics			
Age, years (median, IQR)	68 (60.5–76.5)	69 (65.5–76.5)	0.656
Female, n (%)	18 (36.7%)	24 (45.3%)	0.381
BMI, kg/m^2 (median, IQR)	27.8 (25.1–31.2)	26.2 (23.5–29.0)	0.197
Smoking status, n (%)			
Current	2 (4.1%)	3 (5.7%)	0.899
Ex	22 (51.0%)	22 (41.5%)	
Never	25 (51.0%)	28 (52.8%)	
Aetiology, n (%)			
Idiopathic	36 (73.5%)	24 (45.3%)	**0.004**
Post-infective	3 (6.1%)	9 (17.0%)	0.089
COPD	1 (2.0%)	3 (5.7%)	0.347
Primary ciliary dyskinesia	2 (4.1%)	5 (9.4%)	0.285
Tuberculosis	1 (2.0%)	1 (1.9%)	0.955
Non-tuberculosis mycobacterium	1 (2.0%)	1 (1.9%)	0.955
Inflammatory bowel disease	1 (2.0%)	5 (9.4%)	0.113
Rheumatoid arthritis	3 (6.1%)	1 (1.9%)	0.271
Other	1 (2.0%)	4 (7.5%)	0.198
Comorbidities, *n* (%)			
Cardiac disease	15 (30.6%)	14 (26.4%)	0.639
Stroke	4 (8.2%)	4 (7.5%)	0.908
Chronic renal failure	2 (4.1%)	2 (3.8%)	0.936
Osteoporosis	6 (12.2%)	6 (11.3%)	0.885
Depression	9 (18.4%)	4 (7.5%)	0.102
Anxiety	6 (12.2%)	4 (7.5%)	0.425
Diabetes	5 (10.2%)	5 (9.6%)	0.921
Neoplastic disease	10 (20.4%)	8 (15.1%)	0.482
Clinical status			
BSI score (median, IQR)	6.0 (4.5–8.5)	6.0 (4.0–9.0)	0.849
Exacerbations in previous year, n (%)			
0	10 (20.4%)	10 (18.9%)	0.961
1	11 (22.4%)	13 (24.5%)	
2	10 (20.4%)	9 (17.0%)	
3+	18 (36.7%)	21 (39.6%)	
Hospitalisations in previous year, n (%)			
0	43 (87.8%)	44 (83.0%)	0.500
1+	6 (12.3%)	9 (17.0%)	
Reiff radiology score (median, IQR)	3 (2-5)	3 (2-4)	0.885
Sputum volume (mL)/day (median, IQR)	10 (5–30)	15 (5–30)	0.194
MRC dyspnoea score, n. (%)			
0	6 (12.2%)	7 (13.2%)	0.117
1	14 (28.6%)	21 (39.6%)	
2	13 (26.5%)	7 (13.2%)	
3	6 (12.2%)	13 (24.5%)	
4	10 (20.4%)	5 (9.4%)	
Lung function (median, IQR)			

Continued

Table 3 Continued

Variables	Eosinophilic (BEC ≥300 cells/µL)	Non-eosinophilic (BEC <150 cells/µL)	P value
FEV1 (L)	2.01 (1.53–2.53)	1.89 (1.27–2.62)	0.621
FEV1 % predicted	76.6 (55.2–92.1)	87.0 (54.8–108.5)	0.484
FVC (L)	3.36 (2.65–4.05)	3.12 (2.19–3.85)	0.317
FVC % predicted	98.3 (80.4–111.0)	99.2 (83.0–118.9)	0.841
Aspergillus serology (median, IQR)			
Total IgE, IU/ml	64.5	35.9	**0.034**
Aspergillus-specific IgE, kUA/L	0.03	0.01	**0.005**
Aspergillus-specific IgG, g/L	26.7	28.0	0.625
Treatments, n (%)			
ICS	28 (57.1%)	16 (30.2%)	**0.006**
Macrolide/oral antibiotic	22 (44.9%)	18 (34.0%)	0.258
Airway clearance	37 (75.5%)	40 (75.5%)	0.996

Data presented as median with IQR or n (%) unless otherwise specified.
Pairwise comparisons were performed using Mann-Witney (continuous variables, two groups) or $\chi 2$ (categorical variables).
Bold indicates statistical significance.
BEC, blood eosinophil count; BMI, body mass index; BSI, Bronchiectasis Severity Index; COPD, chronic obstructive pulmonary disease; FEV1, forced expiratory volume in 1 second; FVC, forced vital capacity; ICS, inhaled corticosteroid; MRC, Medical Research Council.

frequencies of the included patients—two factors heavily weighted by the BSI.[19] Our results, however, suggest that eosinophil proteins Gal-10, ECP and EDN inversely correlate with lung function and positively correlate with radiological severity, both of which represent genuine measures of disease severity. While modest, these highly significant correlations suggest that eosinophilic airway inflammation is potentially associated with lung damage/impairment in bronchiectasis, interesting given associations between elevated blood eosinophils and greater lung function decline in the general adult population, independent of asthma and/or smoking status.[24 25] As to why the results for MBP and EPX, despite being the most abundantly expressed proteins in eosinophils,[26] do not reflect that of ECP, EDN and Gal-10 may be explained by lower sensitivity of our assay for these proteins.

In addition, MBP, EDN and ECP are reportedly expressed by basophils, neutrophils and other immune/tissue cells.[26 27] However, given the strong correlations observed between ECP, EDN and both Gal-10 and EPX, two widely recognised eosinophil-specific proteins, it is not expected that the results presented here reflect the actions of other cell types besides eosinophils.

We also report no clear clinical profile associated with eosinophilic disease, including no association between eosinophilic disease and exacerbation frequency as previously documented,[3 4] or an association with lung damage/impairment as identified earlier within the paper when utilising airway levels of eosinophil proteins. We did, however, observe a slight sex disparity, whereby a higher proportion of the eosinophilic group were male. This appears consistent with the sex disparity observed across other eosinophilic conditions, such as hyper-eosinophilic syndrome and eosinophilic oesophagitis, where males are disproportionately affected.[28 29] We also observed a significantly increased frequency of ICS use among those with eosinophilic disease, despite ERS treatment guidelines advising against the use of ICS in those with bronchiectasis without asthma or ABPA (ie, our study population).[30] As blood eosinophilia is a recognised treatable trait in other airway diseases, that clinicians recognise eosinophilia as a steroid-responsive phenotype regardless of the disease classification is somewhat expected or may reflect a lack

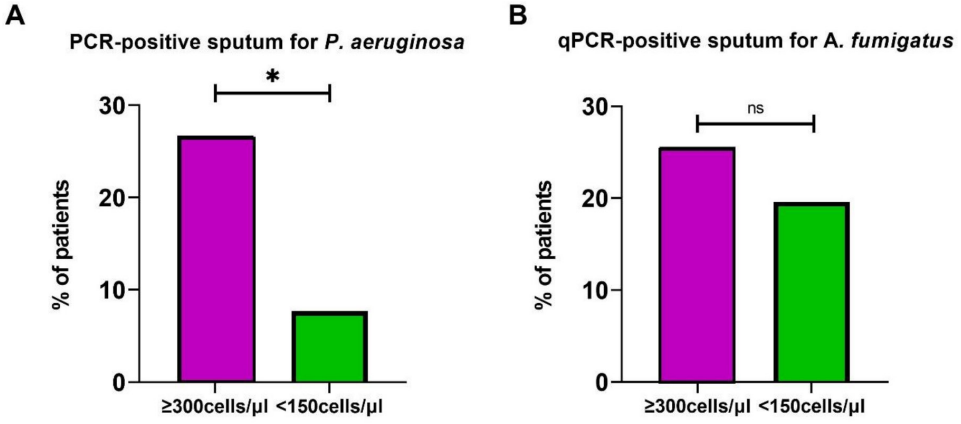

Figure 6 *Pseudomonas aeruginosa* is detected at a higher frequency in those with eosinophilic bronchiectasis (BEC ≥300 cells/µl), unlike A. fumigatus which is detected at a similar frequency in those with eosinophilic and non-eosinophilic disease (BEC <150 cells/µl). Pairwise comparisons were performed using chi-square (categorical variables). BEC, blood eosinophil count; RT-qPCR, real-time quantitative PCR.

of alternative treatment options. Our findings are divergent from recent studies but are highly consistent with the findings from a multicentre European cohort study of 1007 patients showing no increase in BSI or exacerbation rate among those with high BECs ≥300 cells/µL.[3]

Our results align with previous literature confirming BECs as a sufficient, although imperfect, surrogate marker for eosinophilic airway inflammation in bronchiectasis.[3] BECs are known to fluctuate over time[31 32] and in response to internal and external factors.[31–35] Of relevance, studies report that individuals can shift between high and low BEC classifications between subsequent measurements.[31 32] As such, the overlap in airway eosinophil protein levels between BEC groups, the apparent discrepancy in clinical phenotype between those with peripheral blood eosinophilia versus those with elevated levels of eosinophilic airway inflammation, as well as the lack of distinct 'eosinophil-associated' airway inflammatory profile in those with eosinophilic disease defined by BEC may reflect that some individuals are falsely classified as 'eosinophilic' or 'non-eosinophilic' based on BEC. These data emphasise the importance of measuring airway eosinophilia/eosinophilic inflammation when defining eosinophilic disease given the imperfect nature of BECs. Unfortunately, due to a lack of available data, we were unable to further explore this hypothesis, or validate our findings, with the use of sputum eosinophil counts, which may be seen as a limitation of our analysis.

Importantly, the distinct lack of 'eosinophil-associated' airway inflammatory profile, and comparable airway levels of 'neutrophil-associated' inflammatory signatures (including NE, IL-8 and NET immune complexes), in those with eosinophilic disease suggests concurrent neutrophilic inflammation. Therefore, it should be emphasised that many (if not the majority) of individuals with 'eosinophilic bronchiectasis' likely have a mixed eosinophil/neutrophil inflammatory profile, reinforced by the apparent clinical benefit of inhibiting dipeptidyl peptidase-1, an enzymatic protein involved in the activation of neutrophil serine proteases,[36] in those with bronchiectasis with elevated BECs.[37] A mixed inflammatory phenotype may explain the lack of clinical differences observed between BEC groups in our study, as certain clinical parameters (eg, exacerbations) may be primarily neutrophil-driven. This is interesting to consider given that our results suggest associations between airway eosinophil proteins and worsened lung damage/function, perhaps indicating an additive effect of eosinophilic inflammation on these clinical parameters in bronchiectasis.

We also report a significantly increased prevalence of PCR-confirmed airway *P. aeruginosa* in those with eosinophilic disease, interesting as *P. aeruginosa* is a known marker of bronchiectasis severity. Our results extend previous literature showing a *Pseudomonas*-dominant microbiome among those with eosinophilic disease, despite no marked increase in severity. Our finding of higher airway levels of eosinophil proteins in those culturing *P. aeruginosa*, alongside existing literature linking T2-dominant inflammation with *P. aeruginosa* infection in CF [7 8] and murine models,[38 39] further highlights *P. aeruginosa* as a potential microbial driver of eosinophilic disease.

A higher proportion of eosinophilic individuals had serology indicative of *Aspergillus* sensitisation, despite those with ABPA being actively excluded. Severe asthma with fungal sensitisation is a widely recognised clinical phenotype associated with severity in severe asthma and represents an intermediate stage towards ABPA.[40] However, an equivalent phenotype in bronchiectasis is not recognised/diagnosed clinically. Nonetheless, allergic sensitisation is frequent in bronchiectasis in the absence of ABPA, with sensitisation responses, notably those driven by *A. fumigatus*, being associated with worsened lung function, increased disease severity and poor clinical outcomes.[9] Therefore, given the clinical relevance, our data showing a potential link between *Aspergillus* sensitisation and eosinophilic bronchiectasis warrants further investigation.

Our study has the strength of utilising a novel proteomic assay to detect, quantify and assess the clinical significance of eosinophilic airway inflammation in bronchiectasis, something which has historically proven challenging. While previous studies have utilised sputum cell counts to assess eosinophilic airway inflammation, this method often underestimates airway inflammation by only considering intact cells and not those that have lysed, as a consequence of their inflammatory processes or following the various degradation steps during sputum processing. Our airway protein quantification method provides information regarding the activation state of airway eosinophils, unlike the use of BECs and standard sputum cell counts. This study has important limitations. First, it is a single centre study; therefore, the findings require further validation. Second, ICS use may confound the association between eosinophilic inflammation and outcomes; however, sensitivity analysis limited to non-ICS users showed consistent findings. In this study, asthma was excluded based on review by study physicians with access to medical records. However, it should be noted that there is no single test for asthma and therefore excluding asthma in patients with bronchiectasis can be challenging, meaning it is possible that undiagnosed asthma may be present within the cohort. Finally, as our groups appear well balanced with respect to age and sex, it is possible that we have missed important differences between BEC groups given known associations between eosinophilic disease and factors such as sex, severity, aetiology and others. Our sample size was also modest, which may have also influenced our findings.

Interpretation

We report a potential association between eosinophilic airway inflammation and worsened lung function, increased radiological severity, increased *Aspergillus* sensitisation and airway *P. aeruginosa* infection in those with bronchiectasis. BECs are significantly associated with airway eosinophilic inflammation measured using a novel LC-MS/MS-based assay. Our data suggest that direct measurement of eosinophilic airway inflammation in sputum may complement or enhance information obtained from the use of BEC and that inflammatory heterogeneity exists within the airways of those with eosinophilic bronchiectasis.

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