Sputum neutrophil elastase associates with microbiota and *Pseudomonas aeruginosa* in bronchiectasis

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Active neutrophil elastase correlates with low microbiota diversity and *P. aeruginosa* detected as relative abundance (*Pseudomonas*), standard culture and targeted real-time PCR in sputum samples of adult bronchiectasis patients in stable state https://bit.ly/2LUkpVq

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ABSTRACT

Introduction: Neutrophilic inflammation is a major driver of bronchiectasis pathophysiology, and neutrophil elastase activity is the most promising biomarker evaluated in sputum to date. How active neutrophil elastase correlates with the lung microbiome in bronchiectasis is still unexplored. We aimed to understand whether active neutrophil elastase is associated with low microbial diversity and distinct microbiome characteristics.

Methods: An observational, cross-sectional study was conducted at the bronchiectasis programme of the Policlinico Hospital in Milan, Italy, where adults with bronchiectasis were enrolled between March 2017 and March 2019. Active neutrophil elastase was measured on sputum collected during stable state, microbiota analysed through 16S rRNA gene sequencing, molecular assessment of respiratory pathogens carried out through real-time PCR and clinical data collected.

Results: Among 185 patients enrolled, decreasing α-diversity, evaluated through the Shannon entropy (ρ −0.37, p<0.00001) and Pielou's evenness (ρ −0.36, p<0.00001) and richness (ρ −0.33, p<0.00001), was significantly correlated with increasing elastase. A significant difference in median levels of Shannon entropy as detected between patients with neutrophil elastase ⩾20 μg·mL^{-1} (median 3.82, interquartile range 2.20–4.96) *versus* neutrophil elastase <20 μg·mL^{-1} (4.88, 3.68–5.80; p<0.0001). A distinct microbiome was found in these two groups, mainly characterised by enrichment with *Pseudomonas* in the high-elastase group and with *Streptococcus* in the low-elastase group. Further confirmation of the association of *Pseudomonas aeruginosa* with elevated active neutrophil elastase was found based on standard culture and targeted real-time PCR.

Conclusions: High levels of active neutrophil elastase are associated to low microbiome diversity and specifically to *P. aeruginosa* infection.

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Introduction

Bronchiectasis is a chronic respiratory disease characterised by an abnormal and permanent dilatation of the bronchi in the context of a clinical syndrome of cough, sputum production and frequent respiratory infections [1]. Neutrophilic airway inflammation is recognised as one of the major drivers of bronchiectasis pathophysiology, and has clear implications on clinical outcomes [2, 3]. Up to 80% of bronchiectasis patients enrolled across different experiences worldwide show neutrophilic airway inflammation [3–6]. Neutrophil elastase detected in sputum is a promising biomarker in bronchiectasis patients, and its activity correlates not only with disease severity, lung function and quality of life, but also with the exacerbation rate [3].

Neutrophil elastase is released from neutrophils during degranulation and formation of extracellular traps, and has antibacterial effects against respiratory pathogens [7]. Among the different inflammatory stimuli driving the release of neutrophil elastase from neutrophils, bacteria and, specifically, Gram-negative infections (*e.g.*, *Pseudomonas aeruginosa*, *Haemophilus influenzae* and Enterobacteriaceae) are the most potent [3]. Although the association between neutrophil elastase activity (aNE) and the presence of chronic bacterial infection has been widely explored through culture-based microbiology in both bronchiectasis and other chronic respiratory diseases, a deep analysis of the association of airways microbiota and neutrophilic inflammation measured by neutrophil elastase is still unreported in bronchiectasis [3]. Multiple studies enrolling patients with asthma, COPD, idiopathic pulmonary fibrosis or cystic fibrosis demonstrated a loss of bacterial diversity in the lung compared to healthy controls [8–10]. Experiences evaluating airway microbiota did not identify differences in α-diversity between bronchiectasis patients and healthy subjects, although these results are limited by the small sample size and the large heterogeneity that characterises this disease [11–14].

Therefore, we postulated that bronchiectasis patients with elevated aNE comprised a more homogenous group characterised by a distinct microbiome with lower diversity.

Materials and methods

Study design and population

An observational cross-sectional study was carried out at the bronchiectasis programme of the respiratory department, Fondazione IRCCS Ca' Granda Ospedale Maggiore Policlinico, Milan, Italy, between March 2017 and March 2019. Consecutive patients aged ⩾18 years with clinically (daily sputum production) and radiologically (at least one lobe involvement on chest computed tomography) significant bronchiectasis were recruited during clinical stability (⩾1 month from the last exacerbation and antibiotic course). Patients with cystic fibrosis or traction bronchiectasis due to pulmonary fibrosis were excluded. The study was approved by the ethical committee of the hospital, and all subjects provided written informed consent to participate.

Study procedures

Bronchiectasis patients underwent clinical, radiological, microbiological and functional evaluation. Spontaneous sputum samples were obtained and mucus plugs were isolated. DNA was extracted according to a published technique [15, 16]. Positive and negative controls were extracted along with samples. A second aliquot of mucus plug was diluted ×8 in PBS, vortexed and centrifuged at 4°C for 15 min at 3000×*g*. Sample supernatants were stored at −80°C. For aNE assessment, ProteaseTag Active Neutrophil Elastase Immunoassay (Proaxis, Belfast, UK) was used as per manufacturer's instructions [17].

Bacterial real-time PCR and microbiome evaluation

Bacterial DNA was amplified and quantified using real-time PCR assay for *Pseudomonas aeruginosa*, *Staphylococcus aureus*, *Streptococcus pneumoniae* and *Haemophilus influenzae* [18].

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High-throughput sequencing of bacterial 16S rRNA-encoding gene amplicons (V3–V4 region) was performed according to previously published studies [15, 16].

Specific descriptions of real-time PCR procedure and microbiome analysis are reported in the supplementary material.

Clinical variables

Demographics, comorbidities, disease severity, aetiology of bronchiectasis, respiratory symptoms, sputum evaluation, radiological, functional and biological characteristics in stable state were recorded, and are reported in the supplementary material.

Study groups

A priori we split patients into two groups based on the concentration of aNE (20 $\mu g \cdot mL^{-1}$) defined by Chalmers *et al.* [3] as being most associated with worse outcomes in bronchiectasis: aNE $\geqslant$20 $\mu g \cdot mL^{-1}$ *versus* aNE <20 $\mu g \cdot mL^{-1}$.

Statistical analysis

Variables were collected in an *ad hoc* electronic form. Qualitative variables were summarised with absolute and relative (percentage) frequencies, whereas quantitative variables were described using median (interquartile ranges (IQR)) values. Qualitative variables were statistically assessed using Chi-squared or Fisher exact test, when appropriate, whereas those for quantitative nonparametric variables used Mann–Whitney tests. aNE was correlated to continuous variables with Spearman correlation. A two-tailed p-value <0.05 was considered statistically significant. A univariate and multivariate linear regression analysis was carried out to assess the relationship between levels of aNE and epidemiological and microbiological variables based on β-coefficients and related 95% confidence intervals. The statistical software Stata version 15 (StataCorp, College Station, TX, USA) was used for all statistical computations.

Sample size calculation was based on the assumption of a higher level of Shannon diversity in patients with a lower aNE. We assumed a mean difference of 0.5 (means of 2.8 and 3.3 in aNE $\geqslant$20 $\mu g \cdot mL^{-1}$ and aNE <20 $\mu g \cdot mL^{-1}$, respectively) and a common standard deviation of 1.19; the estimated sample size was 180, with an α-error of 0.05 and a statistical power of 0.8.

16S rRNA gene sequences were analysed using the Quantitative Insights into Microbial Ecology (QIIME version 2.0 2019.07 release) pipeline for analysis of microbiome data [19]. Microbiome data are available in the Sequence Read Archive, accession number PRJNA605315. Code and metadata utilised for analysis are available at https://github.com/segalmicrobiomelab/bronchiectasis.neutrophil.elastase. Complete information on bioinformatic and statistical analysis are reported in the supplementary material.

Results

Study population

185 patients (74.6% females, median (IQR) age 63 (51–71) years) were enrolled during the study period. Clinical and microbiological variables are reported in table 1 according to the two study groups. Only five (2.7%) patients of our cohort showed an aNE level below the lower limit of detection. Patients in the aNE $\geqslant$20 $\mu g \cdot mL^{-1}$ group showed higher disease severity, with higher amount of daily sputum production, greater sputum purulence and lower forced expiratory volume in 1 s. >50% of the patients had chronic infection identified by culture, mainly due to *P. aeruginosa* (30.8%), methicillin-sensitive *S. aureus* (9.2%) and *H. influenzae* (8.1%). *P. aeruginosa* was also detected through real-time PCR in 43.2% of the cases, *H. influenzae* in 30.3%, *S. aureus* in 38.4% and *S. pneumoniae* in 36.2%. In terms of microbiome diversity, the median (IQR) Shannon index was 4.33 (3.06–5.60) and evenness was 0.75 (0.59–0.87). 109 (58.9%) patients had a median aNE <20 $\mu g \cdot mL^{-1}$, whereas 76 (41.1%) measured $\geqslant$20 $\mu g \cdot mL^{-1}$.

Microbiome and neutrophil elastase activity

Positive and negative controls were sequenced along with samples. Negative control was reported to have 210 reads that were filtered out during the quality step. Positive control showed *Bacillus* 24%, *Staphylococcus* 12%, *Lactobacillus* 12%, Enterobacteriaceae 11%, *Escherichia* 11%, *Salmonella* 11%, *Listeria* 10% and *Pseudomonas* 9%.

Within-sample microbial diversity, evaluated through the Shannon entropy (ρ −0.37; p<0.00001), Pielou's evenness (ρ −0.36, p<0.00001) and richness (ρ −0.33; p<0.00001), was inversely correlated with values of aNE (figure 1).

Moreover, similar results in within-sample diversity are shown considering the two study groups.

TABLE 1 Clinical characteristics of the study population according to the two study groups

	aNE <20 μg·mL^{-1}	aNE ⩾20 μg·mL^{-1}	p-value
Subjects	109	76	
Male	29 (26.6)	18 (23.6)	0.65
Age years	63 (48–72)	63 (53.5–70.5)	0.81
Smoking status			
Never	57 (52.3)	47 (61.8)	0.18
Former/active	52 (47.7)	29 (38.2)	
Body mass index kg·m^{-2}	21.8 (19–25)	21.0 (19–24)	0.15
Radiology			
Reiff score	4 (3–6)	4.5 (3–9)	0.10
Disease severity			
BSI	7 (4–10)	8 (5–12)	0.03
BSI risk class			
Mild	34 (31.2)	16 (21.1)	0.09
Moderate	40 (36.7)	24 (31.6)	
Severe	35 (31.1)	36 (47.4)	
FACED	2 (1–3)	3 (2–4)	0.02
FACED risk class			
Mild	70 (64.2)	33 (43.4)	0.02
Moderate	29 (26.6)	31 (40.8)	
Severe	10 (9.2)	12 (15.8)	
Comorbidity			
BACI	0 (0–3)	0 (0–0)	0.24
History of pneumonia	73 (67.0)	47 (61.8)	0.47
Gastro-oesophageal reflux disease	48 (44.0)	33 (43.4)	0.93
Rhinosinusitis	43 (35.5)	25 (32.9)	0.36
Cardiovascular diseases	33 (30.3)	27 (35.5)	0.45
Systemic hypertension	24 (22.0)	16 (21.1)	0.88
Asthma	18 (16.5)	8 (10.5)	0.25
Immunodeficiency	17 (15.6)	12 (15.8)	0.97
Osteoporosis	14 (12.8)	13 (17.1)	0.42
Nasal polyposis	14 (12.8)	10 (13.2)	0.95
Previous neoplastic disease	12 (11.0)	14 (18.4)	0.15
COPD	11 (10.1)	7 (9.2)	0.84
Depression	8 (7.3)	9 (11.8)	0.30
History of tuberculosis infection	8 (7.3)	6 (7.9)	0.89
Anxiety	7 (6.4)	4 (5.3)	1.0
Diabetes	5 (4.6)	1 (1.3)	0.40
Pulmonary hypertension	4 (3.7)	3 (4.0)	1.0
Atrial fibrillation	4 (3.7)	4 (5.3)	0.72
Other connective tissue disease	3 (2.8)	1 (1.3)	0.65
Rheumatoid arthritis	2 (1.8)	1 (1.3)	1.0
Stroke	1 (0.9)	1 (1.3)	1.0
Chronic renal failure	1 (0.9)	2 (2.6)	0.60
Aetiology			
Idiopathic	44 (57.9)	68 (62.4)	0.95
Primary ciliary dyskinesia	9 (11.8)	11 (10.1)	
Immunodeficiency	9 (11.8)	14 (12.8)	
Post-infective	7 (9.2)	9 (8.3)	
Other[#]	7 (9.2)	7 (6.4)	
Clinical status			
Sputum volume	10 (5–25)	25 (7–75)	0.0003
>1 hospitalisation previous year	10 (12.8)	14 (19.7)	0.25
Lung function			
FEV_1 % pred	80.6±21.6	76.4±27.3	0.25
FEV_1 <50% pred	6 (5.8)	14 (19.4)	0.005
Standard microbiology			
Chronic infection	41 (40.2)	49 (67.1)	<0.0001
Chronic infection *P. aeruginosa*	24 (23.5)	33 (45.2)	0.003
Chronic infection MSSA	10 (9.8)	7 (9.6)	0.96
Chronic infection *H. influenzae*	11 (10.8)	4 (5.5)	0.28
Chronic infection *S. pneumoniae*	1 (1.0)	1 (1.4)	1.0

Continued

TABLE 1 Continued

	aNE <20 µg·mL^{-1}	aNE ⩾20 µg·mL^{-1}	p-value
Chronic infection MRSA	1 (1.0)	1 (1.4)	1.0
Chronic infection *S. maltophilia*	5 (4.9)	0 (0.0)	0.08
Chronic infection *Achromobacter*	0 (0.0)	3 (4.1)	0.07
Other chronic infection	4 (3.9)	3 (4.1)	1.0
Chronic infection *A. fumigatus*	1 (1.0)	0 (0.0)	1.0
Molecular biology			
Real-time PCR for *Pseudomonas* spp.	35 (32.1)	45 (59.2)	<0.0001
Pseudomonas spp. copies·mL^{-1}, $\log_{10}$	6.9 (5.8–7.5)	8.0 (7.5–8.3)	<0.0001
Real-time PCR for *H. influenzae*	40 (36.7)	16 (21.1)	0.02
H. influenzae copies·mL^{-1}, $\log_{10}$	5.0 (4.3–8.5)	8.4 (4.7–9.0)	0.16
Real-time PCR for *S. aureus*	47 (43.1)	24 (31.6)	0.11
S. aureus copies·mL^{-1}, $\log_{10}$	4.6 (3.5–7.6)	4.0 (3.3–7.2)	0.36
Real-time PCR for *S. pneumoniae*	44 (40.4)	23 (30.3)	0.16
S. pneumoniae copies·mL^{-1}, $\log_{10}$	3.6 (3.2–5.5)	3.3 (2.7–4.7)	0.13

Data are presented as n, n (%), median (interquartile range) or mean±SD, unless otherwise stated. aNE: neutrophil elastase activity; BSI: bronchiectasis severity index; FACED: forced expiratory volume in 1 s (FEV_1), age, chronic colonisation, extension, dyspnoea score; BACI: Bronchiectasis Aetiology Comorbidity Index; *P. aeruginosa*: *Pseudomonas aeruginosa*; MSSA: methicillin-sensitive *Staphylococcus aureus*; *H. influenzae*: *Haemophilus influenzae*; *S. pneumoniae*: *Streptococcus pneumoniae*; MRSA: methicillin-resistant *S. aureus*; *Stenotrophomonas maltophilia*: *S. maltophilia*; *A. fumigatus*: *Aspergillus fumigatus*. #: includes COPD, connective tissue diseases, α_1-antitrypsin deficiency, allergic bronchopulmonary aspergillosis, asthma, cystic fibrosis transmembrane conductance regulator-regulated disorders and aspiration.

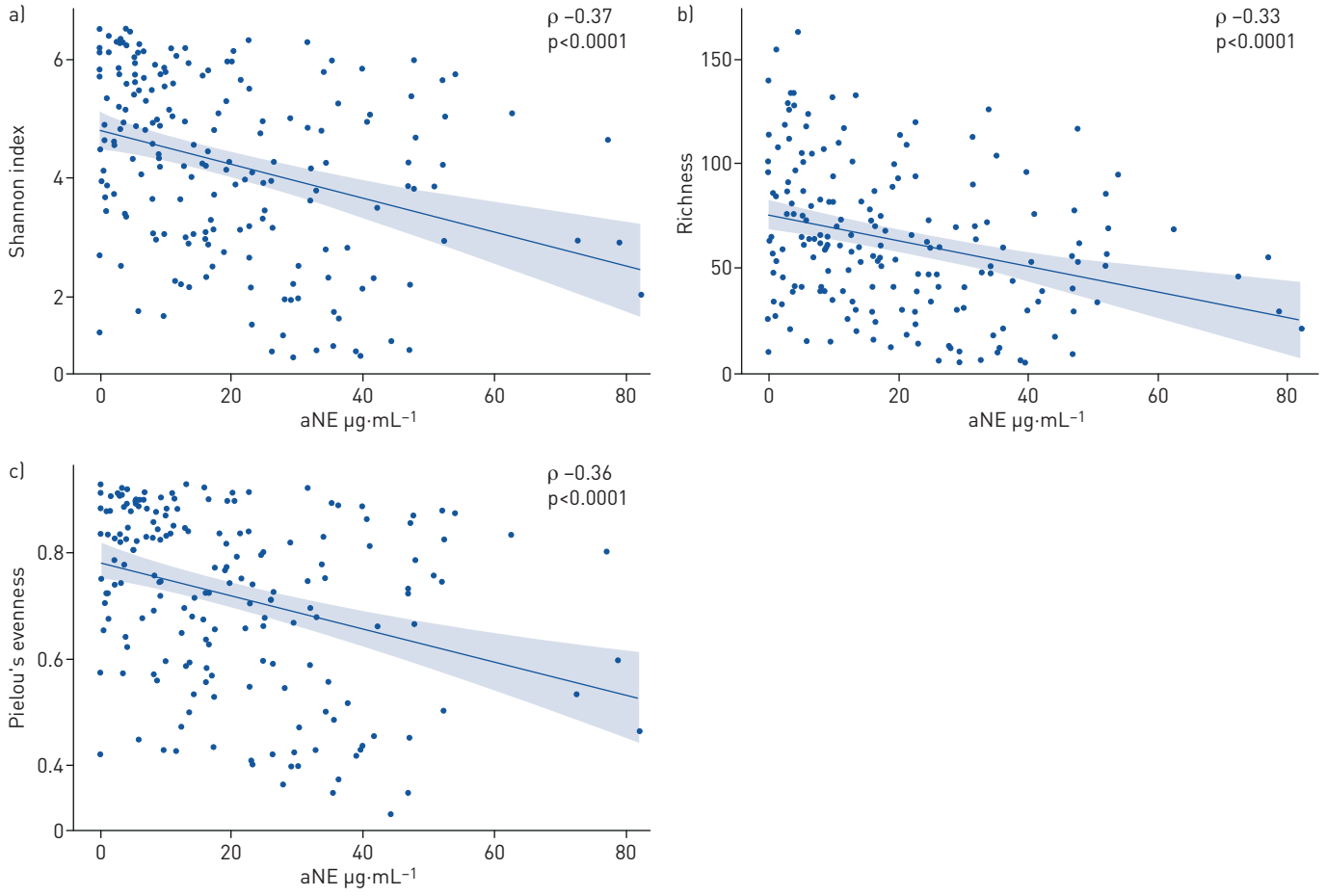

FIGURE 1 Correlation between neutrophil elastase activity (aNE) and a) Shannon entropy, b) richness and c) Pielou's evenness.

A significant difference in terms of median (IQR) levels of Shannon diversity was detected between patients with aNE ⩾20 μg·mL^{-1} (3.82, 2.20–4.96) *versus* aNE <20 μg·mL^{-1} (4.88, 3.68–5.80) (p<0.0001; supplementary figure A). A significant difference was found between patients with aNE <20 μg·mL^{-1} in terms of median (IQR) levels of richness (aNE ⩾20 μg·mL^{-1} 47.0 (29–70.0) *versus* aNE <20 μg·mL^{-1} 65.5 (47.5–96.0); p<0.001) and evenness (aNE ⩾20 μg·mL^{-1} 0.68 (0.47–0.81) *versus* aNE <20 μg·mL^{-1} 0.82 (0.68–0.8878); p<0.0001) (supplementary figure A). Similar results in Shannon diversity were obtained when patients with pulmonary ciliary dyskinesia (PCD) as well as those with primary/secondary immunodeficiency were excluded (supplementary table A). A direct correlation between aNE and *Pseudomonas* relative abundance was found (ρ 0.37, p<0.0001), while an inverse correlation is shown between *Streptococcus* and aNE levels (ρ −0.5, p<0.001). No correlation was seen between *Staphylococcus* and aNE, while a mild inverse correlation emerged with *Haemophilus* (ρ −0.18, p=0.016) (figure 2).

Non-metric multidimensional scaling based on Bray–Curtis dissimilarity is shown in figure 3. Samples clustered in two separate groups according to aNE levels (PERMANOVA p=0.001). Biplot of samples (categorised according to aNE levels) and taxa (summarised at a genus level) showed that the high-aNE group co-occurred with *Pseudomonas*, whereas the low-aNE group co-occurred with *Streptococcus*, *Haemophilus* and *Staphylococcus*.

Further compositional analysis showed a differential enrichment between the two groups: *Pseudomonas* was the only genus increased in the aNE ⩾20 μg·mL^{-1} group, while *Streptococcus*, *Rothia*, *Actinomyces*, *Abiotrophia* and *Atopobium* were differentially enriched among aNE <20 μg·mL^{-1} samples (see volcano plot in supplementary figure B).

Neutrophil elastase activity and chronic bacterial infection at standard microbiology

Chronic bacterial infection and chronic *P. aeruginosa* infection (defined by culture) were more prevalent in patients with aNE ⩾20 μg·mL^{-1} (chronic bacterial infection aNE ⩾20 μg·mL^{-1} 67.1% *versus* aNE

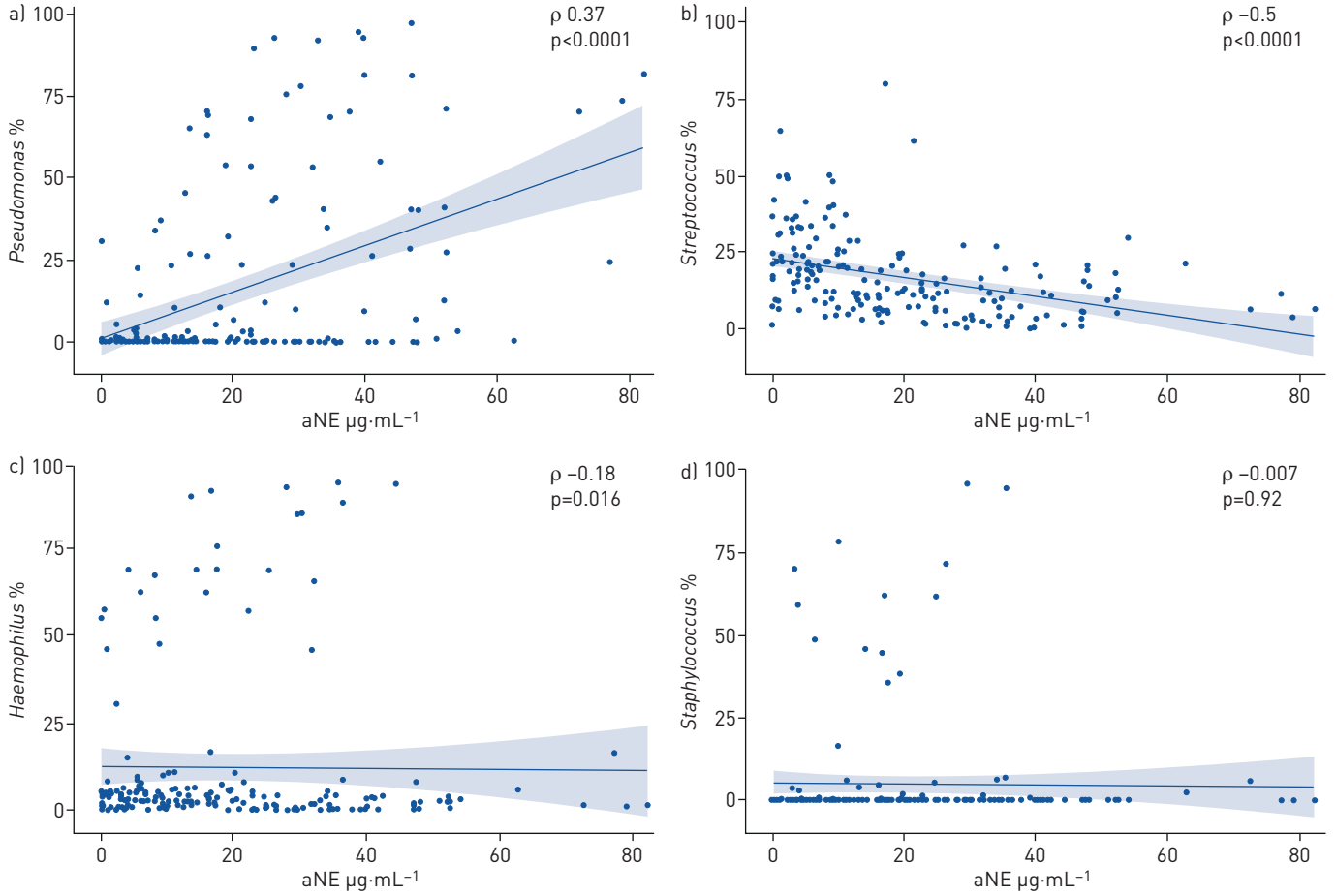

FIGURE 2 Correlation of neutrophil elastase activity (aNE) levels with a) *Pseudomonas*, b) *Streptococcus*, c) *Haemophilus* and d) *Staphylococcus* relative abundance.

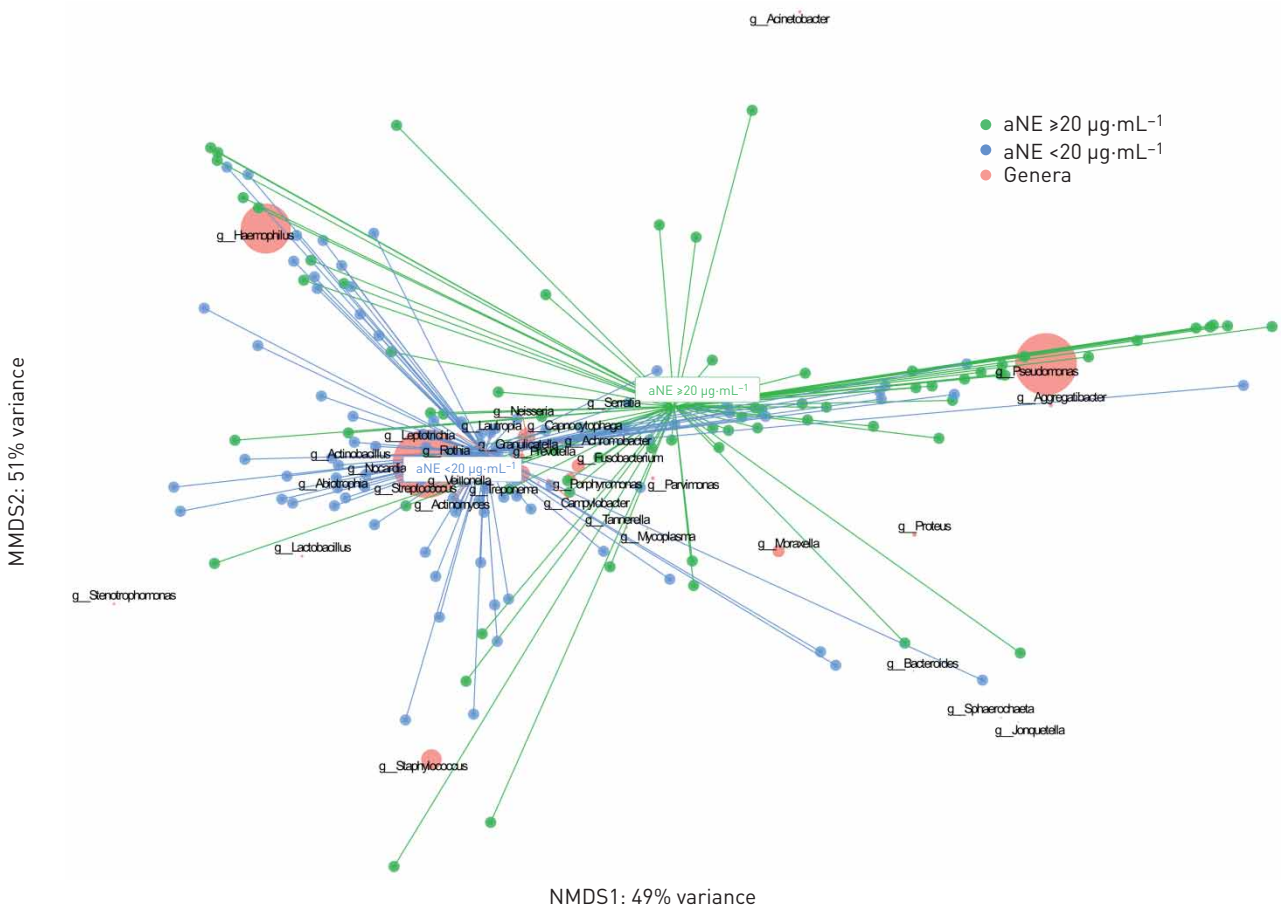

FIGURE 3 Biplot including non-metric multidimensional scaling (NMDS) on the basis of Bray–Curtis distances for groups of neutrophil elastase activity (aNE) ⩾20 µg·mL^{-1} and aNE <20 µg·mL^{-1} and genera.

<20 µg·mL^{-1} 40.2%, p<0.0001; chronic *P. aeruginosa* infection aNE ⩾20 µg·mL^{-1} 45.2% *versus* aNE <20 µg·mL^{-1} 23.5%, p=0.003). No difference in the distribution of chronic infection by *S. aureus*, *H. influenzae*, *S. pneumoniae, Achromobacter denitrificans* was found between the two groups.

Neutrophil elastase activity and molecular biology

A significant correlation was found between aNE and the number of copies·mL^{-1} of *P. aeruginosa* DNA in sputum (ρ 0.42, p<0.0001). Patients with levels of aNE ⩾20 µg·mL^{-1} present with a higher real-time PCR detection in sputum for *P. aeruginosa* (aNE ⩾20 µg·mL^{-1} 59.2% *versus* aNE <20 µg·mL^{-1} 32.1%, p<0.0001). No difference in distribution of infection by *S. aureus* and *S. pneumoniae* was found between the two groups and no correlation was found between aNE and number of copies·mL^{-1} of DNA load for *S. aureus* and *S. pneumoniae*. At real-time PCR, *H. influenzae* was significantly more frequently detected in sputum with lower levels of aNE, while a direct significant correlation was found between aNE and the number of copies·mL^{-1} of *H. influenzae* DNA in sputum (ρ 0.24, p=0.04) (figure 4). A multivariate analysis between *H. influenzae* and *P. aeruginosa* bacterial load is presented in supplementary table B. This multiparametric analysis showed that *H. influenzae* did not correlate with aNE levels. *P. aeruginosa* is the only significant variable associated with aNE (β (95% CI) *P. aeruginosa* 7.8 (0.7–14.9), p=0.04; *H. influenzae* 5.0 (−1.2–11.3), p=0.11).

Discussion

The most important findings of the present study are 1) higher levels of aNE in sputum samples from bronchiectasis patients were correlated with a decreased microbiome diversity; 2) *Pseudomonas* as genus and *P. aeruginosa* as species, detected through both molecular biology and culture, showed the best association with high levels of aNE; and 3) in addition to the presence or absence of *P. aeruginosa* on both culture and molecular biology, high levels of aNE were correlated with the amount of *P. aeruginosa* genome copies in sputum.

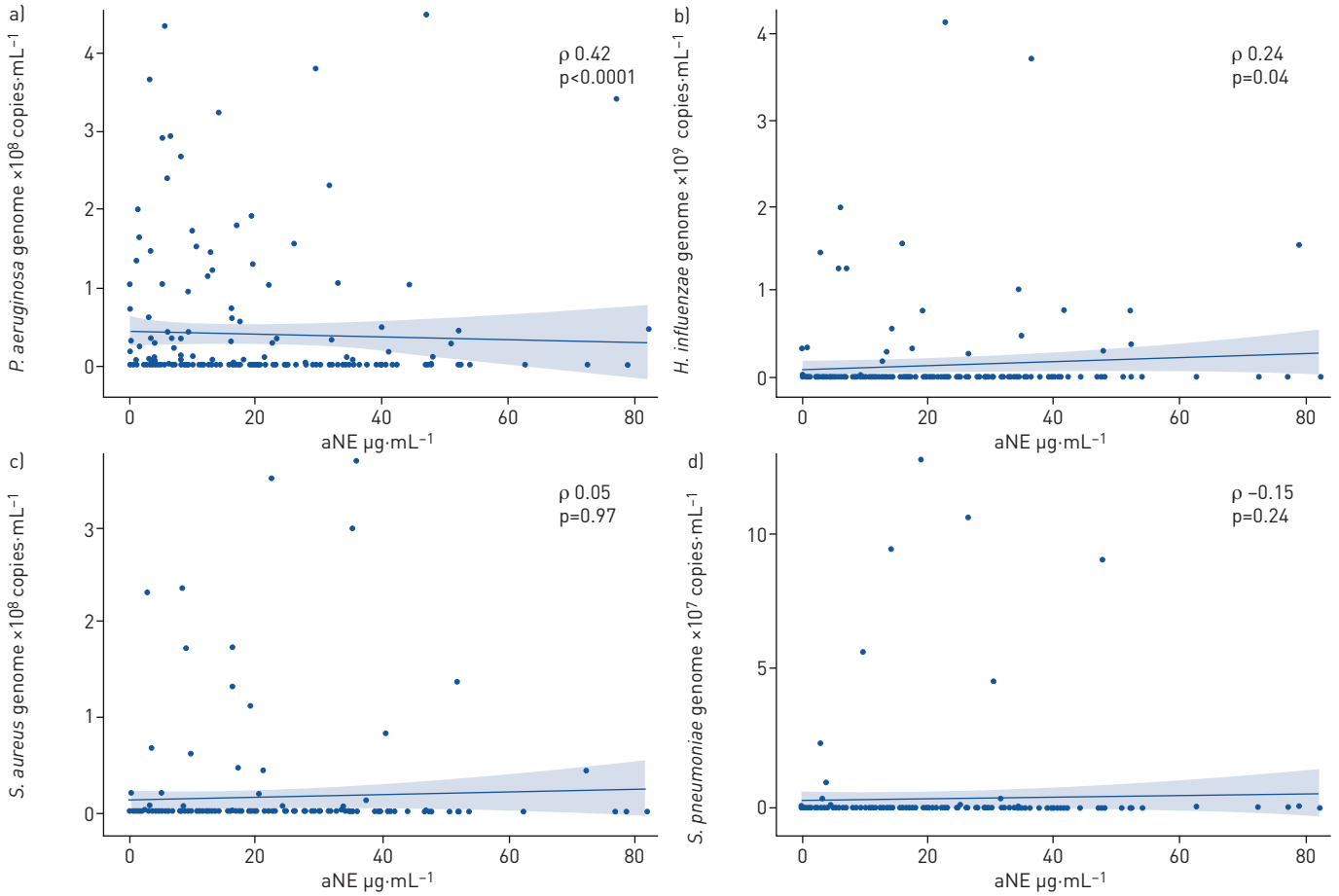

FIGURE 4 Correlation between neutrophil elastase activity (aNE) and number of copies·mL^{-1} of DNA load for a) *Pseudomonas aeruginosa*, b) *Haemophilus influenzae*, c) *Staphylococcus aureus* and d) *Streptococcus pneumoniae*.

As a new finding and in line with our hypothesis, we found an inverse correlation between microbiota diversity and levels of aNE in sputum (based on Shannon, richness and evenness indices). This difference in terms of microbiota diversity was additionally confirmed by the evaluation of the β-diversity, which allowed separating samples in two well-defined groups. The correlation between aNE and a reduced microbiota diversity in sputum is a novel finding in bronchiectasis patients. This is consistent with studies in cystic fibrosis, where Zemanick *et al.* [8] demonstrated that neutrophil elastase levels negatively correlated with microbiota diversity, and reported an association between neutrophil elastase levels and *P. aeruginosa* infection.

From a clinical perspective, the evaluation of aNE in sputum of bronchiectasis patients confirmed data published by Chalmers *et al.* [3], who underlined the correlation between aNE and both disease severity and lung function. From a microbiome perspective, the characteristics of our population were very similar to those reported in previous experiences enrolling adults with bronchiectasis. For example, Lee *et al.* [13] observed that the most abundant phyla among subjects with bronchiectasis are Proteobacteria and Firmicutes. Furthermore, the median levels of aNE in sputum of our patients were very similar to those reported by Chalmers *et al.* [3] in a Scottish population of bronchiectasis patients.

In addition, we confirmed the major role of *P. aeruginosa* in increasing neutrophilic inflammation in bronchiectasis [20]. In the current investigation, the role of *Pseudomonas* as genus and *P. aeruginosa* as species was evaluated not only through microbiome and culture, but also through molecular biology techniques showing a positive correlation between *P. aeruginosa* genome copies·mL^{-1} and aNE. These data support the hypothesis that *P. aeruginosa* appears to be the most potent bacterial stimulus of neutrophil recruitment and release of neutrophil elastase in the bronchiectasis airways. As a new finding, we also discovered that the increased aNE in patients with *P. aeruginosa* was strongly associated with the load of *P. aeruginosa* detected in sputum, opening a new perspective on the role of molecular biology in bronchiectasis. This finding is in line with recent data showing that bacterial load evaluated through

standard microbiology is associated with worse quality of life and increased lung inflammation in bronchiectasis [21]. Further investigations into the role of molecular biology as an aid to define disease severity, outcomes and treatment responses are needed.

Real-time PCR data suggested that the presence of *H. influenzae* was associated with lower levels of aNE, although there was a mild direct correlation between genome copy number and aNE. *H. influenzae* is a Gram-negative bacterium and previous studies described a correlation between chronic infection with *H. influenzae* and higher levels of neutrophil elastase [3]. The increase observed in *H. influenzae* colonised samples seemed to be due to co-infection with *P. aeruginosa*. Multivariate analysis highlighted that *P. aeruginosa* seemed to be the driver of aNE levels even in presence of *H. influenzae* [14]. Our data suggest that the presence of a co-infection with *P. aeruginosa* and its burden are needed to increase levels of aNE in sputum.

With regards to the other genera, Zemanick and co-workers [8, 12] also described a correlation between *Staphylococcus* and Enterobacteriaceae and higher levels of neutrophil elastase in cystic fibrosis, while no association between neutrophil elastase levels and *Haemophilus*, *Prevotella* and *Rothia* was detected. We confirmed the increased enrichment of *Streptococcus* genus associated with low levels of aNE, whereas no evidence of association with *Staphylococcus* was found [22]. Oral commensals are frequently found in healthy sputum microbiota, replaced in disease status with pathogenic bacteria [23].

One of the possible implications of our study is that aNE levels in sputum provide significant insights into the microbial environment in the airways. Microbiota analysis for the assessment of microbial environment in lungs could be used in future clinical trials evaluating the effectiveness of neutrophil elastase inhibitors. Excess of proteases in lungs causes tissue damage and alters the remodelling process. In order to counteract this imbalance, neutrophil elastase inhibitors are currently under clinical development in bronchiectasis [24–26]. Previous and ongoing clinical trials on neutrophil elastase inhibitors were not designed considering bacterial infection with specific pathogens or the presence of active elastase in sputum. The findings presented in the current investigation suggest that presence and burden of *P. aeruginosa* and/or aNE levels could potentially be a useful biomarker where the impact of neutrophil elastase inhibitors could be greatest [24, 25]. Our data are consistent with those of Keir *et al.* [20] and indicate that while patients with chronic *P. aeruginosa* infection may seem to be the best candidates for neutrophil elastase inhibition, its isolation might not be enough to presume high levels of aNE in sputum. Furthermore, the load of *P. aeruginosa* is emerging as a determining factor for the presence of high aNE levels in sputum of bronchiectasis patients and could therefore be used as a biomarker to direct neutrophil elastase inhibition.

One of the major limitations of this study includes the use of sputum as matrix of interest, which is not completely representative of the lower airways. However, sputum sampling is noninvasive and easily accessible and would be preferable as a biomarker that can be repeated multiple times over invasive lower airway samples. Furthermore, we did not assess oral microbiota, a major contaminant of sputum. Future investigations should include oral sampling paired with sputum samples in order to dissect the topographical source of the microbial signatures identified. The monocentric design of this study might limit the generalisability in geographical areas where microbiology could be different. Moreover, viral, mycobacterial and fungal communities were not evaluated. The microbiota analysis through 16S rRNA gene sequencing constitutes a cost-effective approach, but has limited capabilities due to lack of strain level resolution and functional assessment. In addition, our study did not take into account microbiota dynamics and future investigations are needed to both confirm our data and improve our findings with a longitudinal assessment during stable state and exacerbation. Microbial functioning that may affect host immune response and aNE levels should be investigated to better explore host–pathogen interaction in bronchiectasis. In conclusion, aNE levels are inversely associated to within-sample diversity in sputum of bronchiectasis patients, and directly proportional to *P. aeruginosa* relative abundance and load, underling the importance of the assessment of the whole bacterial community. Considering the clinical need for treatment of bronchiectasis patients, it is helpful to understand the microbial environment in lungs, which could both serve as a future biomarker for trials on neutrophil elastase inhibitors or a novel target for personalised approaches.

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