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Choi, Hayoung; Ryu, Soorack; Keir, Holly R.; Giam, Yan Hui; Dicker, Alison J.; Perea, Lidia

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Inflammatory Molecular Endotypes in Bronchiectasis: A European Multicenter Cohort Study

Hayoung Choi[1,2], Soorack Ryu[3], Holly R Keir[1], Yan Hui Giam[1], Alison J Dicker[1], Lidia Perea[1], Hollian Richardson[1], Jeffrey T J Huang[4], Erin Cant[1], Francesco Blasi[5,6], Jennifer Pollock[1], Michal Shteinberg[7], Simon Finch[1], Stefano Aliberti[8,9], Oriol Sibila[10], Amelia Shoemark[1], James D Chalmers[1]

[1]Division of Molecular and Clinical Medicine, University of Dundee, Ninewells Hospital and Medical School, Dundee, United Kingdom

[2]Division of Pulmonary, Allergy, and Critical Care Medicine, Department of Internal Medicine, Hallym University Kangnam Sacred Heart Hospital, Seoul, Republic of Korea

[3]Biostatistical Consulting and Research Lab, Medical Research Collaborating Center, Hanyang University, Seoul, Republic of Korea

[4]Division of Systems Medicine, University of Dundee, Ninewells Hospital and Medical School, Dundee, United Kingdom

[5]Department of Pathophysiology and Transplantation, University of Milan, Milan, Italy

[6]Fondazione IRCCS Ca' Granda Ospedale Maggiore Policlinico, Milan, Italy

[7]Pulmonology Institute and CF Center, Carmel Medical Center and the Technion-Israel Institute of Technology, Haifa, Israel

[8]Department of Biomedical Sciences, Humanitas University, Milan, Italy

[9]Respiratory Unit, IRCCS Humanitas Research Hospital, Milan, Italy

[10]Respiratory Department, Hospital Clínic, IDIBAPS, CIBERES, Universitat de Barcelona, Barcelona, Spain

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Correspondence to: James D. Chalmers, MBChB, PhD, Division of Molecular and Clinical Medicine, University of Dundee, Ninewells Hospital and Medical School, Dundee DD1 9SY, United Kingdom

E-mail: jchalmers@dundee.ac.uk

Author contributions:

Study conception and design: HC, FB, MS, SA, OS, AS, JDC

Data collection: HRK, YHG, LP, HR, EC, JP, SF, SA, OS, AS, JDC

Laboratory work: HRK, YHG, LP, HR, EC, JP, SF, SA, OS, AS, JDC

Data analysis: HC, SR, AJD, JTJH, SA, OS, AS, JDC

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ORCID IDs:

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0000-0003-4812-0653 (H.C.); 0000-0001-7360-6060 (A.S.); 0000-0001-5514-7868 (J.D.C.).

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At a Glance Commentary

Scientific knowledge on the subject

Bronchiectasis is a heterogenous disease. Precision medicine relies on the identification of groups of patients with shared features who may respond more uniformly to treatments. Inflammation and infection are key drivers of bronchiectasis pathophysiology. In addition to neutrophilic inflammation that has classically defined bronchiectasis, eosinophilic inflammation was recently reported in the subset of patients. Regarding infection, the harmful effects of *Pseudomonas aeruginosa* in bronchiectasis are the most well-known. Furthermore, microbiome analysis has provided a more comprehensive profile of bacterial communities in bronchiectasis compared to culture. However, to date few studies have integrated host inflammatory and microbiome data to guide precision medicine in bronchiectasis.

What this study adds to the field

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Using cluster analysis on a large multicenter cohort, we show that bronchiectasis inflammatory endotypes are associated with distinct microbiome profiles and predict risk of exacerbation in patients that are clinically indistinguishable. Sputum and serum inflammatory makers identified four clusters, comprising cluster 1 (milder neutrophilic inflammation), cluster 2 (neutrophilic mixed with type 2 inflammation), cluster 3 (most severe neutrophilic), and cluster 4 (type 2 mixed with epithelial inflammation). The four clusters could not be distinguished by baseline clinical characteristics alone but had distinct microbiome profiles. Proteobacteria and *Pseudomonas* at phylum and genus levels, respectively, were more enriched in clusters 2 and 3 than in clusters 1 and 4. Furthermore, clusters 2 and 3 were significantly associated with increased future exacerbation risk compared to cluster 1, even after taking into account prior exacerbation history. We conclude that increased levels of neutrophilic and type 2 inflammation define endotypes with increased disease activity.

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

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Abstract (247/250 words)

Rationale: Although inflammation and infection are key disease drivers in bronchiectasis, few studies have integrated host inflammatory and microbiome data to guide precision medicine.

Objectives: To identify clusters among bronchiectasis patients based on inflammatory markers and assess the association between inflammatory endotypes, microbiome characteristics, and exacerbation risk.

Methods: Stable bronchiectasis patients were enrolled at three European centers and cluster analysis was used to stratify the patients according to the levels of 33 sputum and serum inflammatory markers. Clusters were compared in terms of microbiome composition (16S rRNA sequencing) and exacerbation risk over 12 months follow-up.

Measurements and Main Results: 199 patients were enrolled (109 [54.8%] female, median age 69 years). Four clusters of patients were defined according to their inflammatory profiles: cluster 1 (milder neutrophilic inflammation), cluster 2 (mixed-neutrophilic and type 2), cluster 3 (most severe neutrophilic), and cluster 4 (mixed-epithelial and type 2). Lower microbiome diversity was associated with more severe inflammatory clusters (P<0.001), and beta-diversity analysis demonstrated distinct microbiome profiles associated with each inflammatory cluster (P=0.001). Proteobacteria and *Pseudomonas* at phylum and genus levels, respectively, were more enriched in clusters 2 and 3 than in clusters 1 and 4. Furthermore, patients in clusters 2 (rate ratio [RR] 1.49, 95% CI 1.16–1.92) and 3 (RR 1.61, 95% CI 1.12–2.32) were at higher risk of exacerbation over 12 months follow-up compared to cluster 1 even after adjustment for prior exacerbation history.

Conclusion: Bronchiectasis inflammatory endotypes are associated with distinct microbiome profiles and future exacerbation risk.

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Keywords: bronchiectasis; microbiome; inflammation; biomarkers; cluster analysis

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Introduction

The most challenging aspect of bronchiectasis management is the disease heterogeneity (1, 2). Although frequently classified as idiopathic or “post-infective”, bronchiectasis is the final common pathway for multiple autoimmune, allergic, infective, and genetic disorders (3, 4). Furthermore, even in patients with the same etiology, great clinical heterogeneity exists. Therefore, precision medicine is fundamental for successful bronchiectasis management, and the development of biomarkers to unveil key pathophysiological processes and guide precision medicine is required (5).

Inflammation and infection are key drivers of bronchiectasis pathophysiology (6). Neutrophilic inflammation is reported in the majority of patients and biomarkers of neutrophilic inflammation have been shown to correlate well with disease severity and long-term outcomes, including future exacerbation risk (7-9). In addition to neutrophilic inflammation, eosinophilic inflammation was recently reported in a subset of approximately 20% of patients with bronchiectasis (10). In patients with bronchiectasis, *Pseudomonas aeruginosa* is the most well-characterized pathogen, and is associated with worse quality of life and increased disease severity such as exacerbations, hospitalizations, and increased mortality (11, 12). Microbiome analysis has allowed a more comprehensive profiling of bacterial communities in bronchiectasis, which revealed reduced diversity and increased relative abundance of Proteobacteria in severe disease (13-15).

In patients with bronchiectasis, clinical trials targeting inflammation or chronic bacterial infection failed to reach their endpoints or showed benefits only in a subset of population (16-18). The results suggest a need to identify groups of patients with shared features (known as “endotypes”) who may respond more uniformly to treatments. Inflammatory endotyping

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using cluster analysis has been successfully applied to other diseases such as asthma and has supported the development of novel anti-inflammatory therapies (19). Inflammatory endotypes of bronchiectasis have not been fully defined (20). To be valid, endotypes should be associated not only with microbiome characteristics but also with exacerbations, the key clinical outcomes for patients with bronchiectasis.

This study aimed to identify clusters of patients based on sputum and serum inflammatory markers and to assess the association between inflammatory endotypes, the microbiome, and clinical outcomes in bronchiectasis.

Methods

Study cohort

This analysis was part of the European Multicentre Bronchiectasis Audit and Research Collaboration (EMBARC)–Bronchiectasis Research Involving Databases, Genomics and Endotyping (BRIDGE) study, a prospective observational cohort study (ClinicalTrials.gov identifier NCT03791086). Patients with stable bronchiectasis were enrolled from three European centers: Fondazione IRCCS Cà Granda Ospedale Maggiore Policlinico University Hospital (Milan, Italy), Hospital de la Santa Creu I Sant Pau (Barcelona, Spain), and Ninewells Hospital (Dundee, United Kingdom) (8, 10). The inclusion criteria were as follows: (1) a previous computed tomography (CT) image showing bronchiectasis; (2) compatible clinical syndrome of cough, sputum (eligible patients could produce spontaneous sputum when stable), and/or recurrent chest infections; (3) a primary diagnosis of bronchiectasis established by a respiratory physician; and (4) clinically stable state. The clinically stable state

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was indicated by the lack of any treatment with antibiotics or oral corticosteroids for pulmonary exacerbation in the previous 4 weeks at the screening visit. The exclusion criteria were as follows: (1) inability to provide informed consent, (2) age < 18 years, (3) active tuberculosis, and (4) bronchiectasis due to cystic fibrosis.

Study measurement

Baseline clinical information collected at enrollment included age, sex, medical history (i.e., symptoms, concomitant medications, and comorbidities), smoking history, spirometry results, radiological distribution of bronchiectasis on the previous CT image, and number of pulmonary exacerbations in the previous year. Disease severity was determined using the Bronchiectasis Severity Index (BSI) (21). Pulmonary exacerbation was defined based on a consensus definition for clinical research in adult patients with bronchiectasis (22).

Sputum and serum samples were obtained to measure inflammatory markers selected to represent the major inflammatory pathways detectable in respiratory samples and have been previously implicated in bronchiectasis (7, 8, 23-28). Twenty sputum and 13 serum inflammatory markers were used, as follows: sputum granulocyte-macrophage colony-stimulating factor (GM-CSF), growth-related oncogene α (GRO-α), interferon (IFN)-γ, interleukin (IL)-1β, IL-2, IL-4, IL-5, IL-6, IL-8, IL-10, IL-13, IL-17, monocyte chemoattractant protein (MCP)-1, neutrophil elastase (NE), neutrophil extracellular trap (NET), pH, resistin, tumor necrosis factor (TNF)-α, thymic stromal lymphopoietin (TSLP), and vascular endothelial growth factor (VEGF) plus serum GM-CSF, GRO-α, IFN-γ, IL-1β, IL-5, IL-6, IL-10, IL-13, IL-17, MCP-1, TNF-α, TSLP, and VEGF. Regarding laboratory assessment, GM-CSF, GRO-α, IFN-γ, IL-1β, IL-5, IL-6, IL-10, IL-13, IL-17, MCP-1, TNF-α, TSLP, and VEGF were measured using a

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multiplex immunoassay (Mesoscale diagnostics, Rockville, MD, USA); IL-2, IL-4, IL-8, and resistin were measured using enzyme-linked immunosorbent assay (R+D systems, Abingdon, UK); and pH was measured by Nano electrode and PH meter (Mettler Toledo, Columbus, OH, USA). Additionally, NET complexes (by Histone-elastase assay) and NE activity measurements were performed, as previously described (7, 8, 29).

Microbiome analysis

DNA was extracted from sputum, and the V3 and V4 regions of the bacterial 16S rRNA gene were sequenced using the Illumina MiSeq platform. The resulting FASTQ files were analyzed using Quantitative Insights in Microbial Ecology, including a quality check for sequencing and contamination errors (13, 30) (see methods and Figure E1 in the online supplement).

Alpha diversity was measured using the Shannon–Wiener Diversity Index (SWDI) to assess within-sample microbiome differences. Beta diversity (between-sample diversity) was calculated using weighted UniFrac and displayed using principal coordinate analysis. PERMANOVA was used to test for significant differences. Linear discriminant analysis effect size and random forest analysis were used to identify taxa that were significantly different between clusters. We used the packages “vegan” and “MicrobiomeAnalystR” in R (version 4.0.4) (31). More detailed information on the microbiome analysis methods has been described in our previous studies (10, 13) and it is also provided as the online supplement.

Statistical analyses

Continuous data are presented as medians with interquartile ranges (IQRs) and the results

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were compared using the Kruskal–Wallis test. Categorical data are presented as numbers (percentages), and the results were compared using Pearson's chi-squared or Fisher's exact test, as appropriate. Statistical significance was set at $P < 0.05$.

After completion of the multiple imputation process (see the online supplement), we performed cluster analysis using the database of 33 inflammatory markers. The optimal number of clusters was predetermined to be "four" by the R (version 4.0.4) package "NbClust," which provides 30 indices for unbiased estimation of the optimal number of clusters within a dataset (32). Out of the 26 indices that could be applied to our dataset, 10 indices indicated that the optimal number of clusters was four (see the online supplement). Clusters, inflammatory molecular endotypes, were generated using the FASTCLUS procedure in SAS (version 9.4) (33, 34). This procedure applies the k-means method of cluster analysis to classify the study population into mutually exclusive groups by comparing the Euclidean distances between each patient and each cluster center in an interactive process.

Univariate and multivariate linear regression analyses were performed to assess the association between inflammatory markers and microbiome characteristics. A subset of data was used for the linear regression analysis as follows: microbiome genera of the top five relative taxa abundance in all populations (independent variables), comprising *Streptococcus*, *Pseudomonas*, *Haemophilus, Veilonella*, and *Prevotella* and six sputum inflammatory markers (dependent variables), comprising sputum IL-1β, TNF-α, NETs, IL-5, VEGF, and GRO-α. Additionally, the model was adjusted for age, sex, inhaled corticosteroids, oral antibiotics, and inhaled antibiotic use. We also performed a negative binomial model to compare future exacerbation frequency over 12 months follow-up among clusters, and the results are reported as rate ratios (RRs) with 95% confidence intervals (CIs). Adjusted analysis was

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performed including prior exacerbations and prior severe exacerbations as independent variables to determine if cluster assignment was independently associated with future exacerbation and severe exacerbation frequency.

Results

Study population

This study initially included 209 patients with available laboratory results for sputum and serum inflammatory markers from the EMBARC–BRIDGE cohort. After excluding 10 patients due to unsuccessful 16S rRNA gene sequencing, we finally included 199 patients in this analysis. The median age of all patients was 69 years (IQR, 61–77 years); 109 (54.8%) patients were female, and most were Caucasian (n = 197, 99%). Among the study population, 84 (41.2%) experienced three or more exacerbations and 26 (13.1%) had a history of hospitalization in the previous year. Regarding disease severity, 27.6%, 35.7%, and 36.7% of the patients comprised the mild, moderate, and severe BSI groups, respectively (Table 1).

Inflammatory endotype cluster profiles

A k-means cluster analysis identified four clusters of patients, as follows: cluster 1 (n = 88, 44.2%), cluster 2 (n = 62, 31.2%), cluster 3 (n = 21, 10.6%), and cluster 4 (n = 28, 14.0%). Figure 1 depicts the comparison of 20 sputum and 13 serum inflammatory markers among the clusters. Cluster 3 was characterized as “most severe neutrophilic inflammation” based on higher levels of IL-1β, IL-8, IL-10, NE, NETs, resistin, and TNF-α; cluster 2 as “mixed-neutrophilic and type 2 (T2) inflammation” based on elevated levels of IL-5, IL-8, NE, and TNF-

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α; cluster 4 as “mixed-epithelial and T2 inflammation” based on elevated GRO-α, IL-5, IL-6, MCP-1, VEGF, and serum IL-17 levels; and cluster 1 as “milder neutrophilic inflammation” based on the lowest levels of inflammatory markers overall. The results of the comparison of all inflammatory markers among clusters are provided in Table E1 in the online supplement. Regarding clinical characteristics, cluster 2 showed more frequent exacerbations (median, 3 [IQR, 1–4]) than the other three clusters (median, 2) (P = 0.030). However, no other baseline clinical characteristics differed significantly among the four clusters. Notably, disease severity was not significantly different between the clusters (Table 1). We also observed no difference in blood eosinophil counts between the clusters. Median blood eosinophils counts were 160 (IQR, 100–283) cells/µl in cluster 1, 160 (100–230) cells/µl in cluster 2, 150 (20–250) cells/µl in cluster 3, and 190 (100–280) cells/µl in cluster 4 (P = 0.18). Inflammatory clusters were present and consistent in all three countries studied (see Table E2 in the online supplement).

Microbiome diversity and structure according to Inflammatory clusters

Lower microbiome diversity, measured using the SWDI, was associated with more severe neutrophilic inflammatory clusters (the lowest alpha diversity was observed in cluster 3, P <0.001). Regarding beta diversity, the four clusters were significantly distinct on a principal coordinate analysis plot (PERMANOVA, P = 0.001) (Figure 2).

The sputum microbiome was more enriched by Proteobacteria in clusters 2 and 3 than in clusters 1 and 4 at the phylum level. Although *Streptococcus*, *Pseudomonas*, and *Haemophilus* were enriched in all clusters at the genus level, the relative taxa abundance significantly differed among the four clusters. The relative abundance of *Pseudomonas* was highest in cluster 3 (mean, 35.9%), followed by cluster 2 (24.3%), cluster 1 (15.9%), and cluster

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4 (10.1%). However, *Streptococcus* was highest in cluster 4 (30.2%), followed by cluster 1 (22.4%), cluster 2 (18.4%), and cluster 3 (10.2%) (Figure 3A and B). Similarly, a linear discriminant analysis effect size analysis showed that *Streptococcus* was associated with cluster 4, *Veillonella* and *Prevotella* were enriched in cluster 1, and *Pseudomonas* in cluster 3. Random forest analysis also revealed that *Prevotella*, *Veillonella*, and *Moryella* were most strongly associated with cluster 1 and that *Streptococcus* was enriched in cluster 4 (Figure 3C and D). Additionally, to minimize the potential effects of antibiotics on microbiome characteristics, a sensitivity analysis was performed in 138 patients, after excluding 61 patients on oral or inhaled antibiotics. The microbiome diversity and structure from the sensitivity analysis were similar to those of the entire study population (see Figure E2 in the online supplement).

Association between microbiome structure and inflammatory markers

A linear regression analysis was performed to further investigate the association between relative taxa abundance at the genus level and inflammatory markers. *Streptococcus*, *Veilonella*, and *Prevotella* were significantly associated with decreased levels of the three neutrophilic inflammatory markers, sputum IL-1β, TNF-α, and NETs, in univariate and multivariate regression analyses. Moreover, *Streptococcus* was significantly related to the increased VEGF and GRO-α levels. In contrast, *Pseudomonas* and *Haemophilus* were significantly associated with increased levels of neutrophilic inflammatory markers (TNF-α and NETs in *Pseudomonas*, IL-1β and TNF-α in *Haemophilus*) in univariate and multivariate regression analyses. *Pseudomonas* was associated with decreased VEGF levels (Table 2). Linear regression analysis choosing independent and dependent variables and *vice versa*

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revealed similar associations between inflammatory markers and the microbiome structure (see Table E3 in the online supplement).

Inflammatory endotypes of bronchiectasis and future exacerbation risk

During 12 months follow-up, there were a total of 321 exacerbations and 46 hospitalizations due to severe exacerbation. Compared with patients of cluster 1, the RR for exacerbation over 12 months follow-up was 1.53 (95% CI, 1.19–1.97) for cluster 2 and 1.46 (95% CI, 1.02–2.09) for cluster 3. Furthermore, compared with patients of cluster 1, the RR for severe exacerbation was 2.53 (95% CI, 1.25–5.14) for cluster 2 and 3.21 (95% CI, 1.35–7.61) for cluster 3. No significant difference was observed between cluster 1 and cluster 4 (Table 3). These results indicate that inflammatory endotypes at baseline were associated with future exacerbation risk. We further investigated whether the inflammatory molecular endotype was independently associated with exacerbation and severe exacerbation risk, after accounting for prior risk of exacerbation which is known to be the strongest predictor of future events. Adjusted analyses confirmed an independent association between cluster 2 and 3 and increased exacerbations during follow-up (Table 3). Figure 4 summarizes the four clusters of bronchiectasis patients according to inflammation and microbiome profile, which is also related to exacerbation risk.

Discussion

In this study, 33 sputum and serum inflammatory markers identified four molecular endotypes in patients with stable bronchiectasis as follows: cluster 1 (milder neutrophilic

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inflammation), cluster 2 (mixed-neutrophilic and T2 inflammation), cluster 3 (most severe neutrophilic), and cluster 4 (mixed-epithelial and T2). The four clusters demonstrated distinct microbiome profiles, including diversity and structure, which was also associated with future exacerbation risk. While previous studies have suggested the relationship between neutrophil inflammatory markers, and blood eosinophil counts, with severity of disease or exacerbations, these markers are not mutually exclusive and therefore it is important to understand how different inflammatory processes interact at the individual patient level. This is the first study the authors are aware of to integrate multiple disease markers to identify clusters, and to demonstrate that these clusters have different clinical outcomes.

Notably, bronchiectasis patients of clusters 2 (mixed-neutrophilic and T2 inflammation) and 3 (most severe neutrophilic) showed significantly higher future exacerbation risk than those of other clusters in this study. Bronchiectasis exacerbation is known to be associated with worse quality of life, more frequent hospitalizations, and increased mortality, indicating the importance of preventing exacerbation in the natural history of bronchiectasis (35, 36). From this view, in this study, inflammatory endotypes of bronchiectasis are valuable in identifying patients with future exacerbation risk and in clearly guiding management plans to prevent exacerbation. Management targeting neutrophilic inflammation include long-term macrolide and potentially anti-inflammatory agents in development such as dipeptidyl peptidase-1 inhibition, and those targeting chronic bacterial infection include inhaled antibiotics (16, 37, 38).

Considering that bronchiectasis is classically regarded as a neutrophilic disease, clusters 1 and 3 may be the most typical inflammatory endotypes in bronchiectasis. When observing clusters 1–3, the more severe the neutrophilic inflammation, the more reduced the

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microbiome diversity was. Furthermore, the more severe the neutrophilic inflammation, the larger the proportion of relative taxa abundance of *Proteobacteria* at the phylum level and *Pseudomonas* and *Haemophilus* at the genus level. Our results may have been inferred from the two conclusions of previous studies. Molecular studies have shown that the degree of neutrophilic inflammation correlates well with disease severity and future exacerbation risk (7, 8), and poor disease outcomes are associated with reduced microbiome diversity and taxa dominated by *Proteobacteria* (13). From this perspective, our study is significant in that we directly demonstrated the association between neutrophilic inflammation and microbiome profile. In this study, linear regression analysis showed that high relative abundance of *Pseudomonas* and *Haemophilus* may shape neutrophilic inflammation in the airway. Consistent with our results, previous studies have reported the induction of different responses by the microbiota. *P. aeruginosa* infection induces neutrophilic inflammation by promoting the release of chemokine (C-X-C) ligand 8, IL-1β, and others (39, 40) and *H. influenzae* is also a potent inducer of NETs (41), whereas *Rothia mucilaginosa* has been reported to exert anti-inflammatory properties (42).

Interestingly, patients in cluster 2 showed neutrophilic and T2 inflammation. This is consistent with a recent study that characterized eosinophilic bronchiectasis (10). Importantly, however, we did not identify a separate T2 high cluster that was separate from neutrophilic inflammation. In asthma, it is common to subdivide patients into T2 high and T2 low, and to regard neutrophilic inflammation as a feature of T2 low disease. Both pure eosinophilic and mixed neutrophilic and eosinophilic profiles are commonly seen in asthma on cell counting (43). Our results suggest a mix of T2 and neutrophilic inflammation is common in bronchiectasis and that most patients with bronchiectasis classified as “eosinophilic bronchiectasis” would have overlapping evidence of neutrophilic inflammation,

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which should be considered when deciding treatment by clinicians. Patients in cluster 2 showed a significantly higher exacerbation frequency than those in other clusters, although cluster 2 revealed lower neutrophilic inflammation (e.g., IL-1β, IL-8, and NETs), less reduced microbiome diversity, and lower taxa abundance of *Pseudomonas* than cluster 3. This result may suggest that a combination of T2 and neutrophilic inflammation is at least as significant in terms of exacerbation risk as severe neutrophilic disease which would be in agreement with a previous study showing that increased blood eosinophil counts were associated with a shortened time to exacerbation even after adjusting for infection status in bronchiectasis (10). However, in patients with mixed-neutrophilic and T2 inflammation endotypes, it may be difficult to define subpopulations who benefit from T2 inflammation-targeting treatments, including inhaled corticosteroids or monoclonal antibodies (44-46). A limitation of our study is a lack of sputum cell counts or fractional exhaled nitric oxide measurements to further characterize T2 inflammation in the clusters. Therefore, future studies are required to establish the definition of the T2 inflammatory endotype in bronchiectasis for the successful management of this inflammatory endotype.

The most unexpected endotype was cluster 4 in this study, which revealed lower neutrophilic but higher macrophage-related, epithelial, and T2 inflammation than the other clusters. The higher macrophage-related inflammation contradicts a previous study reporting that patients with bronchiectasis had a significantly lower rate of macrophages and higher secondary necrotic cells than healthy controls, indicating impaired efferocytosis (clearance of apoptotic neutrophils from the airway), possibly due to the low number of macrophages (26, 27). Cluster 4 also had greater systemic inflammation evidenced by increased serum IL-17. In the airways of patients in cluster 4, macrophages may be viable as a key first-line defense against pathogens and regulate neutrophil numbers (47, 48). Consequently, epithelial

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inflammation was relatively more prominent than neutrophilic inflammation in this population. Our data suggest a subtype of disease with prominent systemic inflammation but indistinct airway infection may benefit more from therapies targeting the epithelium or systemic disease and are less likely to response to antibiotic or airway targeted anti-inflammatory therapies, but future work will be required to understand this group in greater detail.

In this study, clusters 2 and 3, Proteobacteria and *Pseudomonas*-enriched at the phylum and genus levels, respectively, were associated with reduced microbiome diversity and increased risk of exacerbation. In agreement with our results, Dicker et al. revealed that a reduction in microbiome diversity, particularly that associated with *Pseudomonas* dominance, was related to greater disease severity, higher frequency and severity of exacerbations, and higher risk of mortality (13). Although the reduced diversity may be interpreted as the result of increased antibiotic exposure in more severe diseases, this study clarified the reduced microbiome diversity driven by the enrichment of *Pseudomonas* and *Haemophilus* within clusters 2 and 3 in the sensitivity analysis after excluding patients on long-term antibiotics. Hence the relationship between inflammatory cluster and microbiome composition does not appear to be entirely explained by long-term antibiotic use, and the independent relationship between cluster and future exacerbations also suggests that prior antibiotic use does not entirely explain the clusters either. Contrary to our results, Rogers et al. reported that patients with bronchiectasis whose bacterial communities were dominated by *H. influenzae* had significantly fewer exacerbations than those dominated by either *P. aeruginosa* or other species (15). However, recent larger bronchiectasis studies have shown that *Haemophilus*-enriched taxa are associated with more severe neutrophilic inflammation, higher disease severity, and worse outcomes (8, 13). Taken together, these results suggest that *Haemophilus*

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is an important pathogen in bronchiectasis associated with increased inflammation.

At this stage, the microbiome or cytokine levels are not ready for clinical use as biomarkers; therefore, we regard this study as an important proof of concept that inflammatory pathways (endotypes) differ between different groups of patients with bronchiectasis. Bronchiectasis trials have provided heterogeneous results (2), and based on our data, we propose that this is because inflammatory subtypes of the disease exist, and therefore, patients may need targeted treatment. The next step is to operationalize this knowledge by identifying more practical biomarkers that can be used clinically to guide therapy. Currently, direct anti-neutrophils, anti-T2 (anti-IL5 and anti-IL4/IL-13), and more targeted anti-inflammatory and antibiotic treatments are in development for the treatment of bronchiectasis (37, 49, 50). Our study showed how existing neutrophilic (IL-1β, IL-8, IL-10, resistin, and NE) and T2 inflammatory (IL-5) cytokines differentiate into bronchiectasis endotypes, which may also play a major role in guiding the aforementioned novel therapies. Additionally, regarding cytokines such as GM-CSF and VEGF, which have not been extensively investigated in bronchiectasis, this study may be valuable in providing initial steps toward developing biomarkers.

This study has a strength in that this is the first to integrate extensive host inflammatory and microbiome data in bronchiectasis. However, this study has some limitations. First, a relatively small number of patients in clusters 3 and 4 may have prevented meaningful intercluster differences in clinical characteristics at baseline. Second, we used 16S rRNA sequencing, which limits an integrated multi-biome analysis of the bronchiectasis airway by combining bacterial, viral, and fungal community profiles. Third, although sputum sampling is non-invasive and is routinely used in the care of patients with bronchiectasis and in

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microbiome research, it has the recognized limitation of potential contamination from the upper airway. Fourth, as our study aimed to analyze endotypes in patients with stable bronchiectasis, follow-up analyses of changes in inflammatory markers and microbiome composition during exacerbation are warranted. Fifth, this study was conducted in three European countries mostly composed of Caucasians. Thus, future studies to validate our findings should be conducted in other geographical regions or ethnic groups.

In conclusion, sputum and serum inflammatory markers identify four molecular endotypes in patients with stable bronchiectasis. The bronchiectasis inflammatory endotypes are associated with distinct microbiome profiles and future exacerbation risk.

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Figure legends

Figure 1. Heatmaps comparing (A) sputum and (B) serum inflammatory markers among clusters. Scales of heatmaps are set to 10 to 90 percentiles of each value.

The levels of inflammatory markers were compared using the Kruskal–Wallis test, and significantly different values are indicated in bold text. *, $P < 0.05$; **, $P < 0.01$; ***, $P < 0.001$.

GM-CSF, granulocyte-macrophage colony-stimulating factor; GRO-α, growth-related oncogene α; IFN, interferon; IL, interleukin; MCP, monocyte chemoattractant protein; NE, neutrophil elastase; NETs, neutrophil extracellular traps; TNF, tumor necrosis factor; TSLP, thymic stromal lymphopoietin; VEGF, vascular endothelial growth factor.

Figure 2. Microbiome diversity according to the inflammatory endotypes of bronchiectasis. (A) Alpha diversity and (B) beta diversity.

PERMANOVA, permutational multivariate analysis of variance

Figure 3. Microbiome structure according to the inflammatory endotypes of bronchiectasis. (A) A relative taxa abundance at the phylum level, (B) a relative taxa abundance at the genus level, (C) linear discriminant analysis effect size, and (D) random forest analysis.

LDA, linear discriminant analysis

Figure 4. Inflammatory endotypes of bronchiectasis, microbiome profiles, and future exacerbation risk. The plot summarizes four clusters of bronchiectasis according to neutrophilic and type 2 inflammation. Two circles beside each cluster show the relative taxa abundance of Proteobacteria and *Pseudomonas* at phylum and genus levels, respectively, in the sputum microbiome. Clusters 2 and 3 were significantly associated with future exacerbation risk compared to cluster 1.

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Table 1. Baseline clinical characteristics

	Total (n = 199)	Cluster 1 Milder neutrophilic (n = 88, 44.2%)	Cluster 2 Mixed-neutrophilic and type 2 (n = 62, 31.2%)	Cluster 3 Most severe neutrophilic (n = 21, 10.6%)	Cluster 4 Mixed-epithelial and type 2 (n = 28, 14.0%)	*P*-value
Age, years	69 (61–77)	70 (62–77)	68 (62–78)	67 (61–76)	67 (62–76)	0.91
Female sex	109 (54.8)	54 (61.4)	31 (50.0)	10 (47.6)	14 (50.0)	0.42
Inhaled corticosteroid use	113 (56.8)	48 (54.6)	39 (62.9)	12 (57.1)	14 (50.0)	0.65
Oral antibiotic use	46 (23.1)	25 (28.4)	11 (17.7)	6 (28.6)	4 (14.3)	0.27
Inhaled antibiotic use	25 (12.6)	12 (13.6)	6 (9.7)	6 (28.6)	1 (3.6)	0.070
MRC dyspnea scale	2 (1–3)	2 (1–3)	2 (1–3)	2 (2–3)	2 (2–4)	0.19
Smoking status						0.91
Never smoker	117 (58.8)	53 (60.2)	35 (56.5)	14 (66.7)	15 (53.6)	
Ex-smoker	72 (36.2)	32 (36.4)	23 (37.1)	6 (28.6)	11 (39.3)	
Current smoker	10 (5.0)	3 (3.4)	4 (6.4)	1 (4.7)	2 (7.1)	
Smoking pack-years						0.34
<10	12 (6.0)	6 (6.8)	4 (6.5)	0	2 (7.1)	
10–20	19 (9.5)	9 (10.2)	5 (8.1)	3 (14.3)	2 (7.1)	
21–40	24 (12.1)	8 (9.1)	6 (9.7)	3 (14.3)	7 (25.0)	
≥ 40	15 (7.5)	8 (9.1)	5 (8.1)	0	2 (7.1)	
Not reported	12 (6.0)	4 (4.5)	7 (11.3)	1 (4.7)	0	
Exacerbation frequency	2 (1–3)	2 (1–3)	3 (1–4)	2 (1–2)	2 (1–3)	0.035
Exacerbation group						0.17
0	31 (15.6)	15 (17.1)	6 (9.7)	3 (14.3)	7 (25.0)	
1	46 (23.1)	19 (21.6)	13 (21.0)	7 (33.3)	7 (25.0)	
2	40 (20.1)	17 (19.3)	10 (16.1)	7 (33.3)	6 (21.4)	
≥ 3	84 (41.2)	37 (42.0)	33 (53.2)	4 (19.1)	8 (28.6)	
History of hospitalization	26 (13.1)	10 (11.4)	7 (11.3)	4 (19.1)	5 (17.9)	0.59
FEV_1, % predicted						0.49

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≥ 80%	61 (30.7)	32 (36.4)	14 (22.6)	6 (28.6)	9 (32.1)	
50–79%	100 (50.3)	41 (46.6)	36 (58.1)	9 (42.9)	14 (50.0)	
30–49%	31 (15.6)	11 (12.5)	11 (17.7)	4 (19.1)	5 (17.9)	
<30%	7 (3.4)	4 (4.5)	1 (1.6)	2 (9.4)	0	
BSI	7 (4–10)	7 (4–10)	8 (5–11)	8 (4–12)	5 (4–8)	0.15
BSI group						0.46
Mild	55 (27.6)	25 (28.4)	13 (21.0)	6 (28.6)	11 (39.3)	
Moderate	71 (35.7)	32 (36.4)	22 (35.5)	6 (28.6)	11 (39.3)	
Severe	73 (36.7)	31 (35.2)	27 (43.5)	9 (42.8)	6 (21.4)	

Data are presented as medians (interquartile ranges) or numbers (percentages).

MRC, Medical Research Council; FEV_1, forced expiratory volume in 1 s; BSI, Bronchiectasis Severity Index

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Table 2. Association between the relative taxa abundance of top five genera in microbiome and sputum inflammatory markers in patients with bronchiectasis

Independent variable	Dependent variable	Univariate analysis		Multivariate analysis*	
		Coefficient (95% CI)	*P*-value	Coefficient (95% CI)	*P*-value
Streptococcus	IL-1β, pg/mL	–11.3 (–18.5, –4.0)	0.002	–11.9 (–19.3, –4.5)	0.002
	TNF-α, pg/mL	–1.3 (–1.8, –0.7)	<0.001	–1.1 (–1.7, –0.5)	<0.001
	NETs, units/mL	–0.95 (–1.4, –0.5)	<0.001	–0.9 (–1.4, –0.5)	<0.001
	IL-5, pg/mL	–0.006 (–0.2, 0.1)	0.94		
	VEGF, pg/mL	19.7 (6.5, 33.0)	0.004	17.8 (4.2, 31.4)	0.011
	GRO-α, ng/mL	0.1 (0.07–0.19)	<0.001	0.1 (0.07, 0.2)	<0.001
Pseudomonas	IL-1β, pg/mL	1.9 (–2.7, 6.5)	0.42		
	TNF-α, pg/mL	0.7 (0.4, 1.1)	<0.001	0.6 (0.2, 0.98)	0.001
	NETs, units/mL	0.6 (0.3, 0.8)	<0.001	0.5 (0.3, 0.8)	<0.001
	IL-5, pg/mL	–0.04 (–0.1, 0.1)	0.47		
	VEGF, pg/mL	–13.3(–21.5, –5.1)	0.002	–12.7 (–21.5, –3.9)	0.005
	GRO-α, ng/mL	–0.03 (–0.1, 0.01)	0.12		
Haemophilus	IL-1β, pg/mL	15.6 (10.1, 21.1)	<0.001	17.2 (11.6, 22.8)	<0.001
	TNF-α, pg/mL	0.6 (0.2, 1.1)	0.009	0.8 (0.4, 1.3)	0.001
	NETs, units/mL	0.1 (–0.2, 0.5)	0.49		
	IL-5, pg/mL	0.006 (–0.1, 0.1)	0.92		
	VEGF, pg/mL	12.2 (1.5, 22.9)	0.025	10.5 (–0.6, 21.5)	0.06
	GRO-α, ng/mL	0.01 (–0.04, 0.06)	0.74		
Veilonella	IL-1β, pg/mL	–28.3 (–49.1, –7.4)	0.008	–24.7 (–46.0, –3.3)	0.024
	TNF-α, pg/mL	–3.0 (–4.7, –1.4)	<0.001	–2.8 (–4.4, –1.1)	0.001
	NETs, units/mL	–1.7 (–2.9, –0.4)	0.011	–1.3 (–2.6, –0.05)	0.042
	IL-5, pg/mL	–0.1 (–0.6, 0.3)	0.55		
	VEGF, pg/mL	22.6 (–15.9, 61.1)	0.25		
	GRO-α, ng/mL	–0.1 (–0.3, 0.1)	0.300		
Prevotella	IL-1β, pg/mL	–33.4 (–55.2, –11.6)	0.003	–30.7 (–53.1, –8.2)	<0.001

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TNF-α, pg/mL	–3.2 (–4.9, –1.4)	<0.001	–2.9 (–4.6, –1.1)	0.001
NETs, units/mL	–1.9 (–3.2, –0.5)	0.007	–1.4 (–2.7, –0.03)	0.045
IL-5, pg/mL	–0.1 (–0.6, 0.4)	0.64		
VEGF, pg/mL	–8.8 (–49.4, 31.8)	0.67		
GRO-α, ng/mL	–0.03 (–0.2, 0.2)	0.80		

*A multivariate linear regression analysis model was adjusted for age, sex, inhaled corticosteroid use, oral antibiotic use, and inhaled antibiotic use.

CI, confidence interval; IL, interleukin; TNF, tumor necrosis factor; NETs, neutrophil extracellular traps; VEGF, vascular endothelial growth factor; GRO-α, growth-related oncogene α

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Table 3. Unadjusted and adjusted Incident rate ratios for exacerbation frequency over 12 months follow-up according to inflammatory clusters

	Exacerbation		Severe exacerbation	
Unadjusted	IRR (95% CI)	*P*-value	IRR (95% CI)	*P*-value
Cluster 1	1.0 (Reference)		1.0 (Reference)	
Cluster 2	1.53 (1.19–1.97)	0.001	2.53 (1.25–5.14)	0.010
Cluster 3	1.46 (1.02–2.09)	0.039	3.21 (1.35–7.61)	0.008
Cluster 4	1.15 (0.80–1.64)	0.45	1.12 (0.36–3.47)	0.84

	Exacerbation		Severe exacerbation	
Adjusted*	IRR (95% CI)	*P*-value	IRR (95% CI)	*P*-value
Cluster 1	1.0 (Reference)		1.0 (Reference)	
Cluster 2	1.49 (1.16–1.92)	0.002	2.46 (1.21–5.00)	0.013
Cluster 3	1.61 (1.12–2.32)	0.010	2.81 (1.18–6.68)	0.019
Cluster 4	1.28 (0.89–1.83)	0.19	0.99 (0.32–3.07)	0.99

*Adjusted for prior exacerbation history (exacerbation analysis) and prior severe exacerbation (severe exacerbation analysis).

IRR, incident rate ratio; CI, confidence interval

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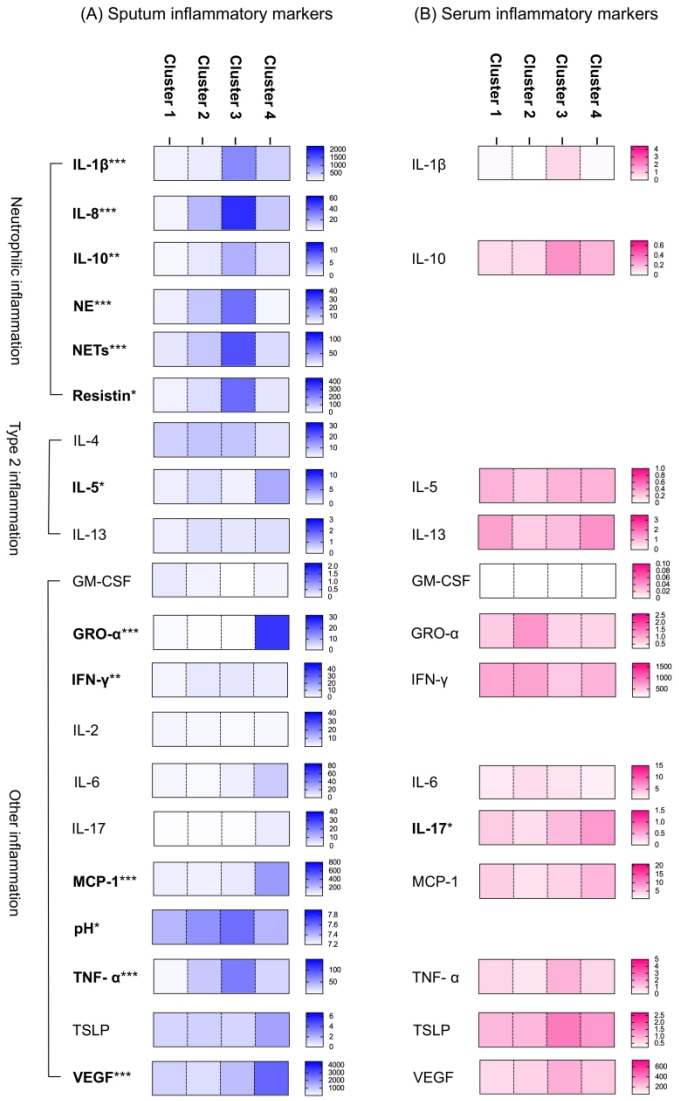

Figure 1. Heatmaps comparing (A) sputum and (B) serum inflammatory markers among clusters. Scales of heatmaps are set to 10 to 90 percentiles of each value.
The levels of inflammatory markers were compared using the Kruskal–Wallis test, and significantly different values are indicated in bold text. *, P < 0.05; **, P < 0.01; ***, P < 0.001.
GM-CSF, granulocyte-macrophage colony-stimulating factor; GRO-α, growth-related oncogene α; IFN, interferon; IL, interleukin; MCP, monocyte chemoattractant protein; NE, neutrophil elastase; NETs, neutrophil extracellular traps; TNF, tumor necrosis factor; TSLP, thymic stromal lymphopoietin; VEGF, vascular endothelial growth factor.

292x450mm (300 x 300 DPI)

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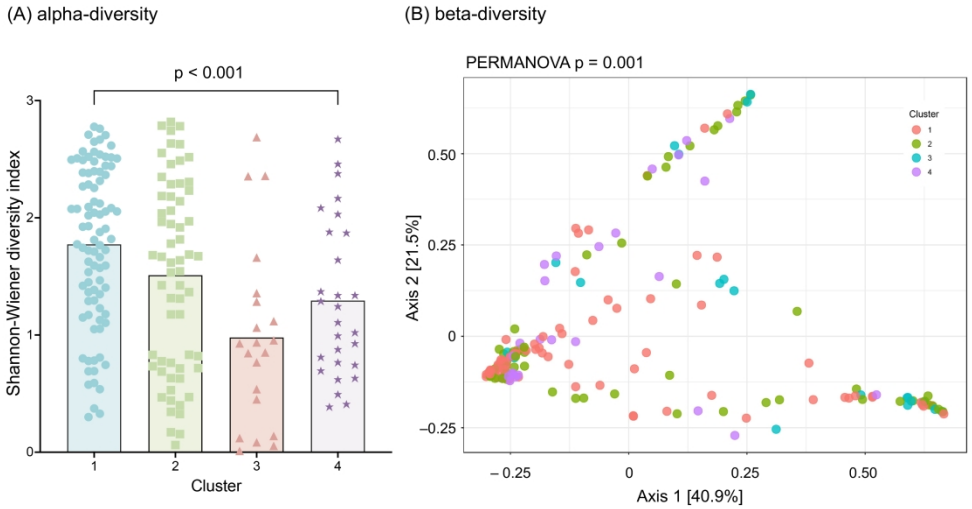

Figure 2. Microbiome diversity according to the inflammatory endotypes of bronchiectasis. (A) Alpha diversity and (B) beta diversity.
PERMANOVA, permutational multivariate analysis of variance

338x181mm (300 x 300 DPI)

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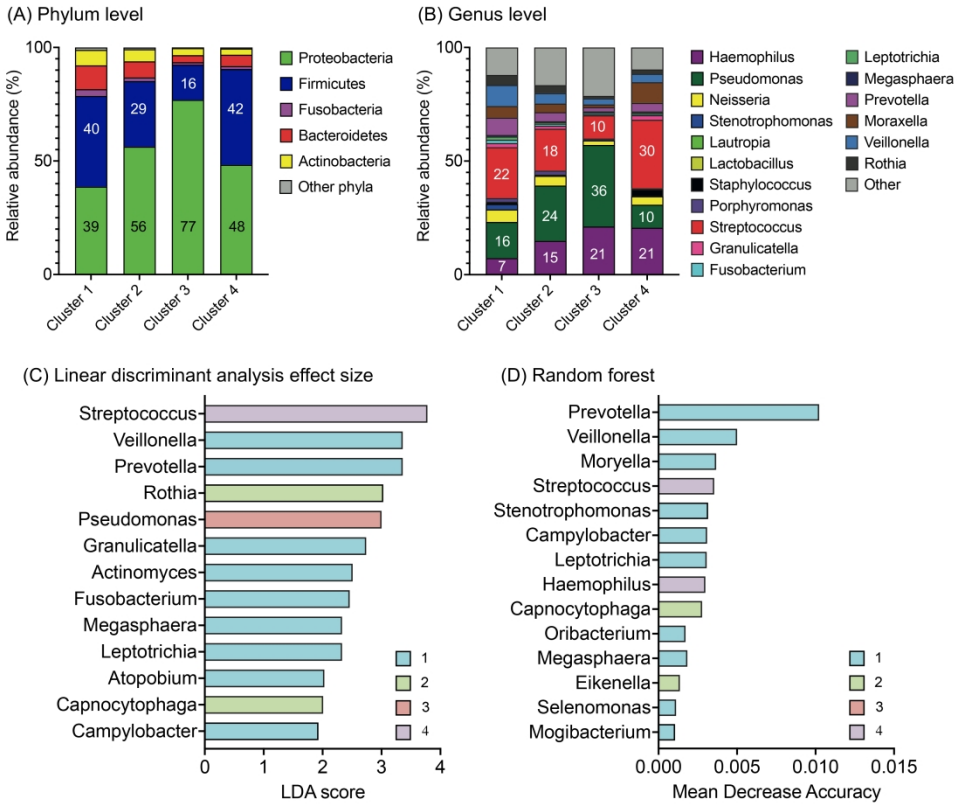

Figure 3. Microbiome structure according to the inflammatory endotypes of bronchiectasis. (A) A relative taxa abundance at the phylum level, (B) a relative taxa abundance at the genus level, (C) linear discriminant analysis effect size, and (D) random forest analysis.
LDA, linear discriminant analysis

338x282mm (300 x 300 DPI)

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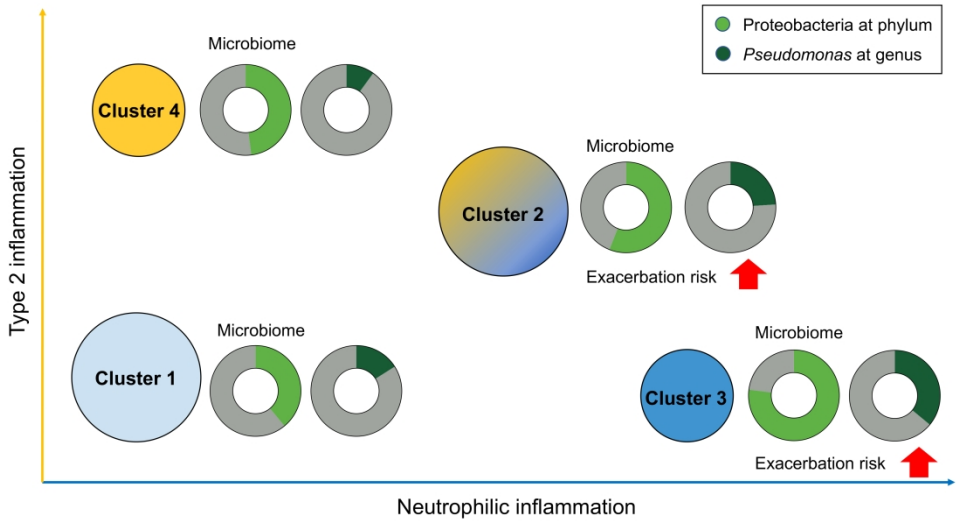

Figure 4. Inflammatory endotypes of bronchiectasis, microbiome profiles, and future exacerbation risk. The plot summarizes four clusters of bronchiectasis according to neutrophilic and type 2 inflammation. Two circles beside each cluster show the relative taxa abundance of Proteobacteria and Pseudomonas at phylum and genus levels, respectively, in the sputum microbiome. Clusters 2 and 3 were significantly associated with future exacerbation risk compared to cluster 1.

331x186mm (300 x 300 DPI)

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Online Data Supplement

Inflammatory Molecular Endotypes in Bronchiectasis: A European Multicenter Cohort Study

Hayoung Choi[1,2], Soorack Ryu[3], Holly R Keir[1], Yan Hui Giam[1], Alison J Dicker[1], Lidia Perea[1], Hollian Richardson[1], Jeffrey T J Huang[4], Erin Cant[1], Francesco Blasi[5,6], Jennifer Pollock[1], Michal Shteinberg[7], Simon Finch[1], Stefano Aliberti[8,9], Oriol Sibila[10], Amelia Shoemark[1], James D Chalmers[1]

[1]Division of Molecular and Clinical Medicine, University of Dundee, Ninewells Hospital and Medical School, Dundee, United Kingdom

[2]Division of Pulmonary, Allergy, and Critical Care Medicine, Department of Internal Medicine, Hallym University Kangnam Sacred Heart Hospital, Seoul, Republic of Korea

[3]Biostatistical Consulting and Research Lab, Medical Research Collaborating Center, Hanyang University, Seoul, Republic of Korea

[4]Division of Systems Medicine, University of Dundee, Ninewells Hospital and Medical School, Dundee, United Kingdom

[5]Department of Pathophysiology and Transplantation, University of Milan, Milan, Italy

[6]Fondazione IRCCS Ca' Granda Ospedale Maggiore Policlinico, Milan, Italy

[7]Pulmonology Institute and CF Center, Carmel Medical Center and the Technion-Israel Institute of Technology, Haifa, Israel

[8]Department of Biomedical Sciences, Humanitas University, Milan, Italy

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[9]Respiratory Unit, IRCCS Humanitas Research Hospital, Milan, Italy

[10]Respiratory Department, Hospital Clínic, IDIBAPS, CIBERES, Universitat de Barcelona, Barcelona, Spain

Correspondence to: James D. Chalmers, MBChB, PhD, Division of Molecular and Clinical Medicine, University of Dundee, Ninewells Hospital and Medical School, Dundee DD1 9SY, United Kingdom

E-mail: jchalmers@dundee.ac.uk

Author contributions:

Study conception and design: HC, FB, MS, SA, OS, AS, JDC

Data collection: HRK, YHG, LP, HR, EC, JP, SF, SA, OS, AS, JDC

Laboratory work: HRK, YHG, LP, HR, EC, JP, SF, SA, OS, AS, JDC

Data analysis: HC, SR, AJD, JTJH, SA, OS, AS, JDC

Writing of the manuscript: HC, JDC

Revising critically for important intellectual content and final approval: all authors

ORCID IDs:

0000-0003-4812-0653 (H.C.); 0000-0001-7360-6060 (A.S.); 0000-0001-5514-7868 (J.D.C.).

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Ingelheim, Chiesi, CSL Behring, Grifols, Insmed, Janssen, Lifearc, and Zambon. JDC is supported by the Asthma and Lung UK Chair of Respiratory Research.

Running head: Inflammatory endotypes of bronchiectasis

Subject category: 10.16: Non-cystic fibrosis bronchiectasis

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Supplementary methods – microbiome analysis

DNA was extracted from 0.1g of whole sputum using the Zymo Quick-DNA Miniprep Plus kit (Zymo Research, Irvine, CA, USA). Samples were processed according to manufacturers' instructions. DNA extraction was performed in batches and an extraction negative controls (100 µl of molecular water-Sigma Aldrich) were performed alongside samples. Metagenomic sequencing of the V3 and V4 region bacterial 16S rRNA gene was performed following the protocol in the Illumina library prep guide (https://www.illumina.com/content/dam/illumina-support/documents/documentation/chemistry_documentation/16s/16s-metagenomic-library-prep- guide-15044223-b.pdf).

Data were quality checked for sequencing and contamination errors. Analysis was conducted in QIIME (Quantitative Insights in Microbial Ecology) version 1.9.0. Sequences were clustered into operational taxonomic units (OTUs) based on 97% sequence similarity using the UCLUST algorithm, aligned against the Greengenes Core reference alignment (Version 13.8) using PyNAST (Version 1.2.2). Taxonomy of the OTUs was assigned using the Ribosomal Database Project Classifier (Version 2.2) with the *de novo* OTU picking option. OTUs were filtered to remove singletons (to improve the accuracy of alpha diversity estimates) and unassigned OTUs, or OTUs identified as Eukaryota, Human and Cyanobacteria. Filtering of OTUs based on those identified as possible contaminants from negative controls (water extracted alongside samples, which mostly produced low read numbers) was applied by comparing the abundance of each OTU in the negative control to the samples; OTUs were removed as contaminants if present in

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similar or greater abundance in the negative controls compared to the samples. A minimum count of reads based on the negative controls was used as a quality control to ensure only those samples with a number of reads above that of the maximum value of the related negative controls. Based on the recommendation from McMurdie & Holme, non-rarefied alpha diversity was measured by determining the Shannon-Wiener Diversity Index in PAST3, whilst beta diversity (between sample diversity) was calculated by weighted UniFrac. All sequences generated are available in the NCBI Sequence Read Archive under the Bioproject accession numbers PRJNA539959, PRJNA548310 and PRJNA316126.

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Supplementary methods – multiple imputation

The dataset of the 33 inflammatory markers had missing values, with a median rate of 18.2% (range, 0–30.6%). Therefore, we imputed missing observations using multivariate imputation by chained equations (MICE). We implemented imputation with 20 repetitions and included all 33 inflammatory markers. Assuming that the likelihood of a missing value is only a function of the observed characteristics, the MICE procedure iteratively estimates the missing values based on Markov Chain Monte Carlo techniques (1, 2). It creates 20 complete datasets to estimate missing values, which are subsequently averaged across all the datasets. We used the package "MICE" in R (version 4.0.4) (3).

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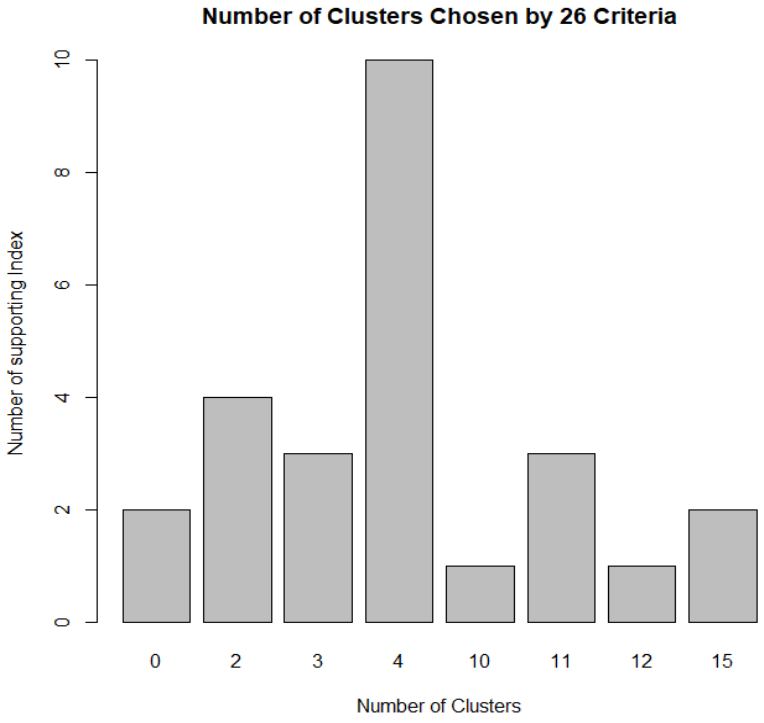
Supplementary methods and results – cluster analysis

The current study employed the "NbClust" package in R (version 4.0.4) to determine the optimal number of clusters for our cytokine dataset (1). The NbClust R package provides 30 indices for an unbiased estimation of the optimal number of clusters within a dataset. Four was chosen as an optimal number of clusters by 10 of 26 indices, which could be applied to our dataset (see the below image). Names of the 26 indices are as follows: "ch," "duda," "pseudot2," "cindex," "beale," "ccc," "ptbiserial," "db," "frey," "hartigan," "ratkowsky," "scott," "marriot," "ball," "trcovw," "tracew," "friedman," "mcclain," "rubin," "kl," "silhouette," "dindex," "dunn," "hubert," "sdindex," and "sdbw."



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An original k-means cluster analysis determined each inflammatory marker as variables 1–33, as shown in below table and performed analysis from variable 1 to variable 33 in numerical order.

Var 1	Var 2	Var 3	Var 4	Var 5	Var 6	Var 7
serum GM-CSF	sputum GM-CSF	serum GRO-α	sputum GRO-α	serum IFN-γ	sputum IFN-γ	serum IL-10
Var 8	Var 9	Var 10	Var 11	Var 12	Var 13	Var 14
sputum IL-10	serum IL-13	sputum IL-13	serum IL-17	sputum IL-17	serum IL-1β	sputum IL-1β
Var 15	Var 16	Var 17	Var 18	Var 19	Var 20	Var 21
serum IL-5	sputum IL-5	serum IL-6	sputum IL-6	sputum IL-8	serum MCP-1	sputum MCP-1
Var 22	Var 23	Var 24	Var 25	Var 26	Var 27	Var 28
serum TNF-α	sputum TNF-α	serum TSLP	sputum TSLP	serum VEGF	sputum VEGF	sputum IL-4
Var 29	Var 30	Var 31	Var 32	Var 33		
sputum IL-2	sputum NETs	sputum pH	sputum resistin	sputum NE		

Var, variable

To evaluate the reproducibility of k-means cluster analysis, we performed 100 simulations with different starting variables and random orders. The table below depicts a part of the simulations with the starting variables and order information.

	Starting variables and orders
simulation 1	var33 var26 var22 var18 var14 var20 var8 var11 var30 var2 var12 var24 var16 var10 var21 var28 var15 var4 var5 var13 var3 var1 var29 var23 var9 var19 var31 var32 var27 var6 var7 var25 var17
simulation 2	var12 var22 var23 var6 var15 var3 var10 var7 var20 var11 var27 var28 var21 var4 var2 var14 var26 var24 var32 var5 var29 var25 var1 var31 var33 var17 var19 var9 var16 var8 var18 var30 var13
simulation 3	var2 var9 var21 var30 var11 var28 var33 var1 var16 var19 var8 var17 var32 var4 var15 var31 var3 var18 var6 var7 var5 var20 var10 var26 var27 var13 var29 var24 var25 var12 var22 var14 var23

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simulation 4	var1 var9 var25 var23 var22 var15 var20 var18 var32 var4 var7 var19 var16 var3 var2 var13 var26 var8 var28 var17 var31 var5 var6 var24 var27 var10 var12 var21 var30 var14 var29 var33 var11
simulation 5	var7 var3 var24 var22 var12 var4 var27 var30 var28 var13 var5 var8 var2 var17 var18 var33 var9 var14 var10 var32 var26 var21 var31 var16 var25 var20 var15 var23 var29 var6 var19 var1 var11
simulation 6	var27 var19 var1 var29 var3 var18 var30 var12 var20 var24 var2 var17 var7 var16 var21 var28 var33 var15 var25 var6 var8 var22 var31 var13 var5 var9 var4 var10 var26 var11 var14 var32 var23
simulation 7	var8 var15 var30 var31 var18 var28 var4 var5 var29 var21 var20 var2 var10 var23 var22 var13 var9 var12 var1 var33 var16 var3 var25 var26 var11 var19 var27 var7 var32 var6 var24 var17 var14
simulation 8	var14 var6 var25 var15 var30 var21 var28 var2 var33 var17 var4 var9 var32 var19 var29 var22 var16 var7 var10 var18 var12 var26 var23 var11 var31 var8 var20 var24 var27 var1 var13 var5 var3
simulation 9	var7 var27 var5 var21 var15 var16 var10 var9 var12 var20 var17 var18 var26 var24 var11 var14 var29 var30 var3 var32 var33 var19 var25 var22 var13 var8 var23 var31 var28 var6 var4 var2 var1
simulation 10	var6 var31 var4 var24 var14 var26 var1 var32 var7 var23 var19 var29 var27 var5 var11 var30 var13 var3 var8 var12 var21 var15 var28 var22 var18 var33 var2 var20 var9 var10 var16 var17 var25
. . . . . .	
simulation 91	var12 var8 var10 var25 var19 var14 var17 var21 var24 var11 var28 var9 var23 var1 var7 var16 var15 var30 var20 var32 var22 var33 var4 var2 var27 var13 var5 var31 var3 var6 var26 var18 var29
simulation 92	var13 var29 var2 var11 var28 var32 var6 var19 var9 var3 var10 var8 var18 var17 var20 var21 var12 var7 var23 var22 var1 var24 var4 var33 var27 var14 var5 var31 var30 var25 var26 var15 var16
simulation 93	var31 var27 var20 var26 var18 var22 var17 var30 var16 var5 var14 var4 var24 var15 var6 var8 var11 var7 var2 var3 var13 var28 var19 var9 var32 var12 var23 var10 var1 var21 var25 var33 var29
simulation 94	var15 var20 var5 var9 var31 var3 var19 var27 var26 var17 var10 var13 var29 var8 var2 var6 var28 var11 var16 var24 var18 var4 var32 var25 var12 var33 var30 var7 var22 var21 var23 var14 var1
simulation 95	var19 var29 var28 var16 var12 var6 var17 var14 var1 var7 var31 var13 var23 var3 var21 var8 var27 var26 var5 var22 var18 var10 var24 var25 var15 var2 var20 var4 var9 var11 var32 var30 var33

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simulation 96	var30 var21 var27 var11 var9 var29 var10 var19 var31 var23 var20 var1 var3 var14 var28 var13 var6 var24 var2 var4 var26 var18 var8 var5 var7 var25 var22 var16 var33 var32 var17 var12 var15
simulation 97	var13 var26 var5 var17 var2 var9 var24 var19 var6 var10 var31 var3 var22 var32 var12 var20 var7 var15 var25 var14 var33 var21 var4 var30 var16 var18 var23 var28 var29 var8 var1 var27 var11
simulation 98	var14 var12 var10 var28 var6 var26 var17 var27 var32 var31 var22 var30 var4 var13 var5 var20 var19 var7 var33 var2 var3 var11 var21 var15 var24 var16 var18 var23 var9 var8 var29 var25 var1
simulation 99	var28 var4 var17 var11 var5 var1 var33 var31 var26 var21 var20 var18 var8 var14 var22 var19 var7 var16 var24 var6 var13 var30 var25 var23 var27 var29 var3 var2 var12 var9 var15 var32 var10
simulation 100	var26 var21 var13 var1 var33 var10 var23 var17 var28 var2 var6 var3 var27 var20 var11 var15 var5 var14 var30 var8 var16 var32 var7 var12 var24 var19 var4 var29 var31 var18 var22 var25 var9

Var, variable

A reproducibility between clusters acquired by the original k-means cluster analysis and cluster sets made by the 100 simulations was assessed by the Adjusted Rand Index. The Adjusted Rand Index is a measure of the similarity between two clusterings and has a value between 0 and 1, with 0 indicating that the two data clusterings do not agree on any pair of points and 1 indicating that the data clusterings are the same; negative values can be yielded if the index is less than the expected index (2,3). As all the 100 simulations showed 1 on the Adjusted Rand Index compared with the original clusters, we concluded that our k-means analysis was reproducible.

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Supplementary methods – analytic codes

<R codes>

```
###install packages

install.packages("mice")

install.packages("VIM")

install.packages("miceadds")

install.packages("flexclust")

install.packages("NbClust")

install.packages("factoextra")

install.packages("cluster")


###load packages

library(mice)

library(VIM)

library(miceadds)

library(flexclust)

library(NbClust)

library(factoextra)

library(cluster)


###(MICE analysis) open dataset

setwd("H:/stats/37_HC")
```


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```
data=read.csv("data.csv", header=TRUE)
summary(data)

###(MICE analysis) Multiple Imputation
imp=mice(data, m=20, printFlag=FALSE, maxit=50, seed=1234)
fit1=with(imp,
        exp=lm(y~var1+var2+var3+var4+var5+var6+var7+var8+var9+var10+var11+var12+var13+
        var14+var15+var16+var17+var18+var19+var20+var21+var22+var23+var24+var25+var26
        +var27+var28+var29+var30+var31+var32+var33))

pooled=pool(fit1)
print(pooled)
summary(pooled)

###(MICE analysis) export data
data_final=complete(imp,10)
summary(data_final)
write.csv(data_final,"data_final.csv")

###(NbClust analysis) open dataset
setwd("H:/stats/37_HC")
data=read.csv("data_final_m20.csv", header=TRUE)
```


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```
summary(data)


###(NbClust analysis) optimal number of clusters
set.seed(4648)
res=NbClust(data = data, distance = "euclidean", min.nc = 2, max.nc = 15, method = "kmeans")
barplot(table(res$Best.nc[1,]), xlab="Number of Clusters", ylab="Number of supporting Index",
        main = "Number of Clusters Chosen by 26 Criteria")


<SAS codes>
##open dataset;
OPTIONS VALIDVARNAME = any;


PROC IMPORT
        DATAFILE = "H:\stats\37_HC\data_final_m20.xlsx" OUT = a DBMS = EXCEL REPLACE;
        GETNAMES = YES;
        SHEET = "Sheet1";
RUN;


*k-means cluster analysis;
PROC FASTCLUS DATA = a LIST MAXITER = 10 MAXCLUSTERS = 4 OUT = result; VAR var1-var33;
        RUN;
```


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Figure E1. Stacked microbiomes showing the profiles of the negative controls analyzed at Phyla and Genera level. Details of the numbers of reads obtained from the negative controls and samples are shown in the table.

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Number of samples	199
Sample Reads	
Minimum	2878
Mean	70291
Maximum	310688
Number of negative controls	24
Negative controls Reads	
Minimum	1
Mean	788
Maximum	5745

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Figure E2. Microbiome diversity and structure according to the inflammatory endotypes of bronchiectasis in a subgroup of patients who were not on oral or inhaled antibiotics. (A) Alpha diversity, (B) beta diversity, (C) relative taxa abundance at the phylum level, (D) relative taxa abundance at the genus level, (E) linear discriminant analysis effect size, and (F) random forest analysis.
PERMANOVA, permutational multivariate analysis of variance.

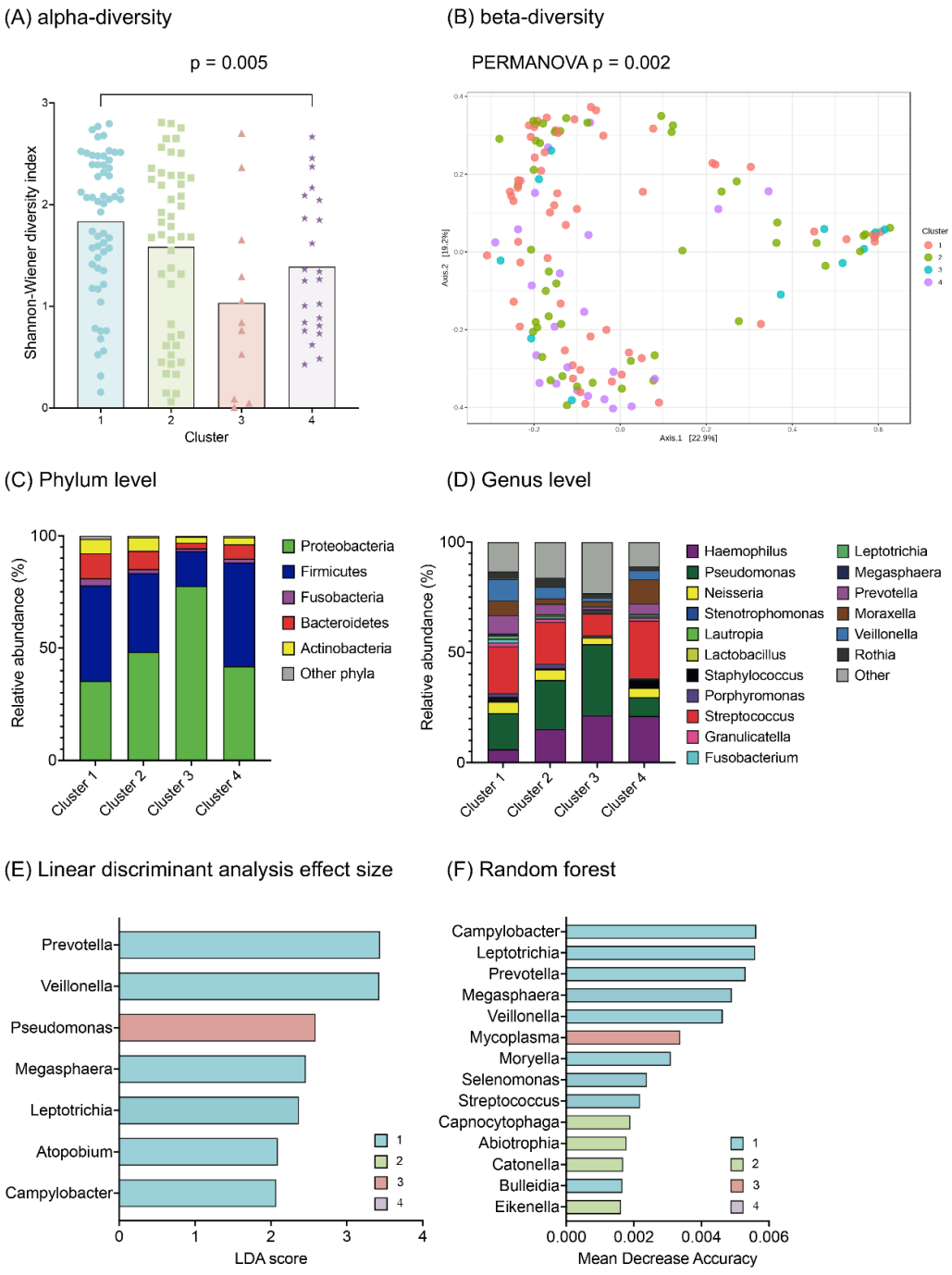

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Table E1. Comparison of sputum and serum inflammatory markers among clusters

	Cluster 1 Milder neutrophilic (n = 88, 44.2%)	Cluster 2 Mixed-neutrophilic and type 2 (n = 62, 31.2%)	Cluster 3 Most severe neutrophilic (n = 21, 10.6%)	Cluster 4 Mixed-epithelial and type 2 (n = 28, 14.0%)	*P*-value
Sputum inflammatory markers					
GM-CSF, pg/mL	0.2 (0–0.4)	0.1 (0–0.4)	0 (0–0.3)	0.1 (0–0.4)	0.33
GRO-α, ng/mL	0.8 (0–3.9)	0.1 (0–0.7)	0.1 (0–0.6)	25.3 (21.6–34.2)	<0.001
IFN-γ, pg/mL	2.0 (0.2–7.8)	4.6 (1.1–13.9)	4.8 (2.2–15.3)	3.5 (2.0–54.3)	0.009
IL-1β, pg/mL	124.7 (36.1–479.3)	188.9 (47.5–1044.7)	1035.3 (323.7–2668.8)	418.7 (175.1–1364.1)	<0.001
IL-2, pg/mL	1.9 (0.2–89.0)	1.4 (0.3–6.9)	1.2 (0.4–60.9)	1.4 (0.3–57.6)	0.97
IL-4, pg/mL	7.0 (2.8–17.6)	8.6 (3.5–17.8)	8.6 (5.3–16.9)	4.9 (2.6–13.1)	0.27
IL-5, pg/mL	0.8 (0.2–2.5)	1.6 (0.5–5.5)	0.7 (0.4–3.1)	4.0 (0.5–11.9)	0.018
IL-6, pg/mL	3.3 (1.1–14.2)	2.1 (0.2–7.2)	5.6 (0.8–14.4)	17.3 (4.8–45.3)	0.001
IL-8, ng/mL	2.9 (0.8–6.9)	17.5 (12.6–20.0)	52.7 (41.0–64.6)	13.9 (6.2–18.9)	<0.001
IL-10, pg/mL	0.4 (0.1–1.7)	1.2 (0.2–4.6)	4.0 (1.4–8.8)	1.5 (0.3–16.8)	<0.001
IL-13, pg/mL	0.2 (0.0–1.3)	0.4 (0.2–1.8)	0.3 (0.2–2.4)	0.4 (0.2–1.8)	0.14
IL-17, pg/mL	0.2 (0–3.0)	0.3 (0–16.0)	0.4 (0–33.4)	3.0 (0–20.3)	0.42
MCP-1, pg/mL	56.8 (11.7–182.4)	48.3 (14.8–165.7)	74.1 (28.7–181.0)	316.7 (164.0–938.6)	<0.001
NE, µg/mL	2.9 (0.7–8.4)	9.4 (1.6–26.4)	23.7 (0.7–4.9)	1.9 (0.7–4.9)	<0.001
NETs, units/mL	15.7 (7.4–42.1)	30.9 (11.9–77.3)	86.6 (32.7–142.5)	20.9 (5.8–84.5)	<0.001
pH	7.4 (7.3–7.6)	7.5 (7.4–7.8)	7.6 (7.4–7.7)	7.4 (7.2–7.6)	0.017
Resistin, ng/mL	24.5 (3.4–115.0)	58.9 (9.6–255.5)	260.7 (32.0–421.4)	44.3 (13.5–225.2)	0.015
TNF-α, pg/mL	4.6 (1.0–34.9)	31.2 (5.5–66.9)	73.7 (24.5–106.0)	24.0 (6.9–79.0)	<0.001
TSLP, pg/mL	1.1 (0.4–3.0)	1.2 (0.5–2.7)	1.1 (0.5–2.5)	2.4 (0.7–5.0)	0.15
VEGF, pg/mL	812.8 (399.6–1651.0)	611.0 (73.7–1012.1)	1161.1 (300.9–2414.7)	2678.3 (911.2–3657.1)	<0.001
Serum inflammatory markers					
GM-CSF, pg/mL	0 (0–0)	0 (0–0)	0 (0–0.1)	0 (0–0)	0.39
GRO-α, ng/mL	0.7 (0.4–1.6)	1.2 (0.7–2.0)	0.6 (0.5–1.2)	0.6 (0.3–1.2)	0.06
IFN-γ, pg/mL	4.5 (2.2–10.1)	3.0 (1.3–15.1)	4.0 (1.6–6.3)	6.2 (3.1–9.5)	0.65
IL-1β, pg/mL	0.1 (0–0.3)	0 (0–0.2)	0.7 (0.1–2.4)	0.1 (0–0.2)	0.45

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IL-5, pg/mL	0.3 (0.1–0.6)	0.2 (0.1–0.7)	0.3 (0.2–0.5)	0.3 (0.1–0.6)	0.69
IL-6, pg/mL	2.0 (1.1–4.2)	2.7 (1.2–6.2)	2.2 (0.9–4.9)	1.6 (1.0–3.0)	0.73
IL-10, pg/mL	0.1 (0–0.3)	0.1 (0–0.3)	0.3 (0.1–0.6)	0.2 (0–0.4)	0.19
IL-13, pg/mL	1.3 (0–2.7)	0.7 (0–2.3)	0.9 (0–1.9)	1.5 (0–2.8)	0.92
IL-17, pg/mL	0.3 (0–1.4)	0.2 (0–2.2)	0.4 (0.1–4.1)	0.6 (0.2–1.3)	0.041
MCP-1, pg/mL	660.5 (361.7–1036.0)	689.4 (298.7–1160.3)	472.0 (291.7–731.0)	599.4 (432.5–1012.2)	0.21
TNF-α, pg/mL	0.8 (0.2–1.4)	0.5 (0.2–1.5)	1.5 (0.9–2.4)	0.8 (0.2–1.1)	0.71
TSLP, pg/mL	0.9 (0.5–1.9)	0.9 (0.3–1.9)	1.5 (1.0–2.4)	1.2 (0.5–1.9)	0.21
VEGF, pg/mL	158.4 (109.3–315.5)	172.3 (113.8–405.7)	267.0 (134.9–415.8)	200.1 (106.7–373.1)	0.40

Data are presented as medians and interquartile ranges.

GM-CSF, granulocyte-macrophage colony-stimulating factor; GRO-α, growth-related oncogene α; IFN, interferon; IL, interleukin; MCP, monocyte chemoattractant protein; NE, neutrophil elastase; NETs, neutrophil extracellular traps; TNF, tumor necrosis factor; TSLP, thymic stromal lymphopoietin; VEGF, vascular endothelial growth factor

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Table E2. Composition of enrollment centers in each inflammatory cluster

	Cluster 1	Cluster 2	Cluster 3	Cluster 4	Total
Center 1	39 (47.6)	20 (24.4)	7 (8.5)	16 (19.5)	82
Center 2	36 (43.4)	24 (28.8)	12 (14.5)	11 (13.3)	83
Center 3	13 (38.3)	18 (52.9)	2 (5.9)	1 (2.9)	34

Data are presented as numbers and percentages.

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Table E3. Association between sputum inflammatory markers and the relative taxa abundance of top five genera in microbiome in patients with bronchiectasis

Independent variable	Dependent variable	Univariate analysis		Multivariate analysis*	
		Coefficient (95% CI)	*P*-value	Coefficient (95% CI)	*P*-value
IL-1β, pg/mL	*Streptococcus*	–0.004 (–0.007, –0.001)	0.002	–0.004 (–0.007, –0.002)	0.002
	Pseudomonas	0.002 (–0.002, 0.006)	0.42		
	Haemophilus	0.009 (0.006, 0.012)	<0.001	0.009 (0.006, 0.012)	<0.001
	Veilonella	–0.001 (–0.002, –0.0003)	0.008	–0.001 (–0.002, –0.0001)	0.024
	Prevotella	–0.001 (–0.002, –0.0005)	0.003	–0.001 (–0.002, –0.0003)	0.008
TNF-α, pg/mL	*Streptococcus*	–0.070 (–0.101, –0.038)	<0.001	–0.064 (–0.097, –0.031)	<0.001
	Pseudomonas	0.104 (0.053, 0.154)	<0.001	0.084 (0.033, 0.136)	0.001
	Haemophilus	0.054 (0.014, 0.095)	0.009	0.074 (0.032, 0.115)	0.001
	Veilonella	–0.021 (–0.032, –0.009)	<0.001	–0.020 (–0.031, –0.008)	0.001
	Prevotella	–0.019 (–0.030, –0.009)	<0.001	–0.018 (–0.029, –0.007)	0.001
NETs, units/mL	*Streptococcus*	–0.091 (–0.133, –0.050)	<0.001	–0.093 (–0.136, –0.050)	<0.001
	Pseudomonas	0.137 (0.070, 0.204)	<0.001	0.127 (0.060, 0.194)	<0.001
	Haemophilus	0.019 (–0.035, 0.073)	0.49		
	Veilonella	–0.020 (–0.035, –0.005)	0.011	–0.016 (–0.032, –0.001)	0.042
	Prevotella	–0.020 (–0.034, –0.001)	0.007	–0.015 (–0.030, –0.0004)	0.045
IL-5, pg/mL	*Streptococcus*	–0.005 (–0.133, 0.124)	0.94		
	Pseudomonas	–0.075 (–0.281, 0.104)	0.47		
	Haemophilus	0.008 (–0.153, 0.169)	0.92		
	Veilonella	–0.014 (–0.059, 0.032)	0.55		
	Prevotella	–0.010 (–0.053, 0.033)	0.64		
VEGF, pg/mL	*Streptococcus*	0.002 (0.001, 0.004)	0.004	0.002 (0.0004, 0.003)	0.011
	Pseudomonas	–0.004 (–0.006, –0.001)	0.002	–0.003 (–0.005, –0.001)	0.005
	Haemophilus	0.002 (–0.0001, 0.004)	0.06		
	Veilonella	0.0003 (–0.0002, 0.001)	0.25		
	Prevotella	–0.0001 (–0.001, 0.0003)	0.67		

*A multivariate linear regression analysis model was adjusted for age, sex, inhaled corticosteroid use, oral antibiotic use, and inhaled antibiotic use.

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CI, confidence interval; IL, interleukin; TNF, tumor necrosis factor; NETs, neutrophil extracellular traps; VEGF, vascular endothelial growth factor; GRO-α, growth-related oncogene α

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