[Chest Infections Original Research]

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A Cluster Analysis of Bronchiectasis Patients Based on the Airway Immune Profile

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BACKGROUND: Clinical heterogeneity in bronchiectasis remains a challenge for improving the appropriate targeting of therapies and patient management. Antimicrobial peptides (AMPs) have been linked to disease severity and phenotype.

RESEARCH QUESTION: Can we identify clusters of patients based on the levels of AMPs, airway inflammation, tissue remodeling, and tissue damage to establish their relationship with disease severity and clinical outcomes?

STUDY DESIGN AND METHODS: A prospective cohort of 128 stable patients with bronchiectasis were recruited across three centers in three different countries (Spain, Scotland, and Italy). A two-step cluster strategy was used to stratify patients according to levels of lactoferrin, lysozyme, LL-37, and secretory leukocyte protease inhibitor in sputum. Measurements of inflammation (IL-8, tumor growth factor β, and IL-6), tissue remodeling and damage (glycosaminoglycan, matrix metallopeptidase 9, neutrophil elastase, and total and bacterial DNA), and neutrophil chemotaxis were assessed.

RESULTS: Three clusters of patients were defined according to distinct airway profiles of AMPs. They represented groups of patients with gradually distinct airway infection and disease severity. Each cluster was associated with an airway profile of inflammation, tissue remodeling, and tissue damage. The relationships between soluble mediators also were distinct between clusters. This analysis allowed the identification of the cluster with the most deregulated local innate immune response. During follow-up, each cluster showed different risk of three or more exacerbations occurring ($P = .03$) and different times to first exacerbations ($P = .03$).

INTERPRETATION: Bronchiectasis patients can be stratified in different clusters according to profiles of airway AMPs, inflammation, tissue remodeling, and tissue damage. The combination of these immunologic variables shows a relationship with disease severity and future risk of exacerbations.

CHEST 2021; ■(■):■-■

KEY WORDS: airway immunity; bronchiectasis; cluster analysis; exacerbations

ABBREVIATIONS: AMP = antimicrobial peptide; GAG = glycosaminoglycan; IQR = interquartile range; SLPI = secretory leukocyte protease inhibitor; TGF-β = tumor growth factor β

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FUNDING/SUPPORT: This study is supported by Instituto de Salud Carlos III and Fondos FEDER [Grant PI18/00311] and Sociedad Española de Neumología y Cirugía Torácica (SEPAR). This work was performed within the Ph.D. Immunology Program of Universitat Autònoma de Barcelona.

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DOI: https://doi.org/10.1016/j.chest.2020.11.011

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Take-home Point

STUDY QUESTION: Can we identify distinct clusters of bronchiectasis patients based on airway immune profile?
RESULTS: We found three clusters of patients based on airway AMPs, inflammation, tissue remodeling, and tissue damage associated to distinct severity disease and future exacerbations.
INTERPRETATION: The combination of airway AMPs, inflammation, tissue remodeling, and tissue damage markers shows a relationship with disease severity and future risk of exacerbations.

Bronchiectasis in adults is a heterogeneous, chronic, irreversible airway disease characterized by recurrent airway infection that worsens the prognosis.[1] Clinical heterogeneity in airway diseases has been studied from clinical data.[2,3] In bronchiectasis, chronic infection with *Pseudomonas aeruginosa*, other pathogens, and daily sputum production without airway infection allowed the identification of four clinical phenotypes.[2] However, a need exists to identify biological clusters based on pathobiological mechanisms (endotypes) to target antiinflammatory therapies better.

Neutrophilic inflammation is one of the major drivers in bronchiectasis. Neutrophils are recruited to the lung during infection in proportion to bacterial load.[4] In the lung, neutrophils release antimicrobial peptides (AMPs), also produced by alveolar macrophages and airway epithelial cells.[5,6] To our knowledge, the most abundant and relevant AMPs in other chronic airway diseases and in *P. aeruginosa* infection are LL-37, secretory leukocyte protease inhibitor (SLPI), lactoferrin, and lysozyme.[7-9] Recently, we showed that bronchiectasis patients demonstrated deregulated airway AMP levels, especially the frequent exacerbator phenotype.[10] Elevated LL-37 and reduced SLPI levels are related independently with more severe disease and can predict risk of exacerbations. Furthermore, elevated LL-37, lactoferrin, and reduced SLPI are associated with airway infection, especially that resulting from *P. aeruginosa*.[10] Airway AMPs in COPD are associated with inflammatory markers such as IL-6, IL-8, and tumor necrosis factor α.[11] However, the relationships between airway AMPs and cytokines have not been described yet in bronchiectasis.

Airway epithelial damage is another consequence of neutrophilic inflammation, which favors airway infection.[12] Among the constituents of the airway epithelium, glycosaminoglycans (GAGs) are polysaccharides expressed ubiquitously on the extracellular matrix of the lung and cell surface and in intracellular compartments.[13] GAGs also can interact and modulate the function of AMPs,[14] DNA, chemokines, cytokines, growth factors, enzymes, and adhesion molecules.[15,16] To our knowledge, GAGs levels in bronchiectasis have not been described yet.

Biological heterogeneity in airway diseases is reported in COPD and asthma. Biologic clusters based on sputum IL-1β, serum C-X-C motif chemokine 10, and the number of peripheral eosinophils allow for the recognition of clinical COPD exacerbation phenotypes.[17] Furthermore, sputum cellular and cytokines profiles are associated with distinct and overlapping groups of patients with asthma and COPD.[18,19] This clustering strategy therefore could be a potential tool to discriminate patients better.

In this study, we hypothesized that the distinct degrees of disease severity may be explained by profiles of airway AMPs, inflammation, tissue remodeling, and tissue damage. First, we identified clusters of patients based on airway AMP levels; second, we showed that clusters were linked to airway inflammation, remodeling, and tissue damage; and finally, we found that these clusters differ in clinical severity and outcomes.

Methods

Study Design and Ethics

This international, multicenter, prospective study included consecutive adult patients with bronchiectasis. The study was approved by the ethics committee “Comitè Ètic d’Investigació Clínica de la Fundació de Gestió Sanitària de l’Hospital de la Santa Creu i Sant Pau de Barcelona” (Identifier: IIBSP-BRO-2013). All patients signed the informed consent form.

Patients

Clinically stable patients with bronchiectasis (n = 128) were recruited from three regional specialist bronchiectasis clinics: Hospital de Sant Pau i la Santa Creu (Barcelona, Spain), Ninewells Hospital (Dundee, Scotland), and Ospedale Maggiore Policlinico (Milano, Italy). Inclusion and exclusion criteria were defined as previously described.[7] Demographic and clinical characteristics were recorded. To ascertain the reference AMP levels in indications not associated with bronchiectasis, we included eight control participants with no

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respiratory conditions and normal spirometry results. For neutrophil experiments, 10 healthy donors, sex- and age-matched with patients, were included. Patients were followed-up prospectively for 1 year to assess the number and the time of the first exacerbation from the inclusion.

Sample Collection and Processing

Spontaneous sputum samples were obtained at the time of inclusion and were processed within 2 h of collection as described previously.[10] Bacteriologic assays were performed, and sputum supernatants were frozen at -80°C until analysis.

Bacteriology Analysis

Specific microorganisms were identified in sputum samples according to standard laboratory methods and were classified as recognized pathogenic bacteria—*P. aeruginosa*, *Haemophilus influenzae*, *Streptococcus pneumoniae*, *Moraxella catarrhalis*, *Stenotrophomonas*, *Serratia*, *Staphylococcus* species, and *Escherichia coli*—or nonpathogenic bacteria—*Streptococcus viridans*, *Corynebacterium* species, and coagulase-negative staphylococci.[20]

AMPs and Cytokine Measurements

Sputum LL-37 (Hycult Biotech), lactoferrin, lysozyme (AssayPro), SLPI (R&D Systems), IL-8, tumor growth factor β (TGF-β; Mabtech AB), and IL-6 (Immunotools) were measured by commercial enzyme-linked immunosorbent assay kits.[7] The limits of detection were 10 pg/mL for IL-8 and IL-6 and 40 pg/mL for TGF-β. Samples were diluted 1:25 for IL-8, 1:5 for TGF-β, and 1:10 for IL-6.

Tissue Remodeling and Damage Assessment

Sulphated GAGs (keratan, chondroitin, and heparin sulphate) were measured in sputum supernatants using a commercial competitive enzyme-linked immunosorbent assay detection kit (Fine Biotech Co.). The limit of detection was 1.563 ng/mL, and samples were diluted 1:3. Levels of matrix metallopeptidase 9 were measured using a commercial enzyme-linked immunosorbent assay kit (R&D Systems). Neutrophil elastase activity was measured by activity-based immunoassay (ProAxsis Ltd.), as described previously.[21]

DNA Measurement

Total DNA was extracted from sputum supernatant using the QIAmp DNA Blood Kit (Qiagen) following the manufacturer's instructions and was measured using the Nanodrop ND-1000 Spectrophotometer (Thermo Fisher Scientific). Bacterial DNA was isolated using the Femto Bacterial DNA Quantification Kit (Zymo Research) by a quantitative real-time polymerase chain reaction system (ThermoFisher).

Blood Neutrophil Chemotaxis Assays

Roswell Park Memorial Institute 1640 medium (Lonza), supplemented with 10% sputum supernatants, was placed in the bottom of a 24-well plate with 3-µm transwell inserts (Millipore). Above, 1×10^6/mL of healthy blood neutrophils purified using Ficoll-Hypaque, dextran-saccharose sedimentation, and lysis of erythrocytes were added and incubated for 4 h at 37°C 5% CO_2. Next, inserts were removed and the migrating neutrophils at the bottom were harvested and counted by flow cytometry (MACSQuant cytometer; Miltenyi Biotec). As a control for unspecific migration, a well with neutrophils and medium alone was included.

Clustering Analysis

To identify the phenotypes of bronchiectasis based on sputum AMPs, we carried out a scalable two-step cluster analysis using log-likelihood distance measures. The clustering was an explorative analysis to group patients based in the distribution of LL-37, SLPI, lactoferrin, and lysozyme concentrations. The sequence of the process was: (1) testing the normal (Gaussian) distribution of variables by the Kolmogorov test, (2) analyzing the independency of the variables by bivariate correlation and validating it by the scatterplot of the pairs of variables, and (3) an unsupervised two-step clustering using log-likelihood distance measurement and continuous variables standardized using *Z* scores in SPSS version 22 software (SPSS Inc.). This clustering method is preferred when the most appropriate number of clusters to fit the data is not known before the clustering procedure. Two clustering variables (LL-37 and SLPI) finally were selected because they did not have substantial multicollinearity ($r > 0.3$) among the four AMPs (correlation matrix in e-Table 1). The optimal number of clusters (n = 3) was selected automatically by an algorithm based on Akaike's information criterion. The resulting clusters distributed the patients in cluster 1 (n = 33), cluster 2 (n = 74), and cluster 3 (n = 21). The two variables included produced a silhouette coefficient of 0.7, indicative of good data partitioning. In this model, we found that SLPI showed a predictive strength of 1 and LL-37 showed a predictive strength of 0.99 in accordance with cluster ability and quality. To minimize order effects, we randomly ordered the patients for the analysis.

Statistical Analysis

The Kolmogorov-Smirnov test was applied to test for normal data distribution. Categorical variables were presented as frequencies. Continuous variables were presented as mean and SD or median and interquartile range (IQR; 25th-75th percentiles). The comparisons were analyzed using the analysis of variance or their corresponding nonparametrical tests when required. Multiple comparisons between groups were analyzed by the Bonferroni or Dunn test, according to their normal distribution. Correlations were analyzed using Pearson or Spearman coefficients according to their normal distribution. Time to first exacerbation was modeled using Kaplan-Meier analysis. In each figure legend, the number of samples is indicated. The correlation matrix was obtained by R software (R Foundation for Statistical Computing) and Corrplot packages.[22] A *P* value of less than .05 was considered significant.

Results

Clinical Characteristics

Table 1 shows the demographic and clinical characteristics of all patients. The mean age was 69 ± 10 years and 56.3% were women. Patients were predominantly nonsmokers (59.4%) and had idiopathic bronchiectasis (45.3%). The mean FEV_1 (percentage predicted) was 78.4 ± 28.8 L and the mean BMI was 25.7 ± 5.5 kg/m^2. Half of the patients experienced frequent exacerbations.[23] Demographics and clinical characteristics of control participants are shown in e-Table 2.

Compared with control participants, bronchiectasis patients showed significantly higher sputum levels of LL-37 (median, 0.02 µg/mL [IQR, 0.01-0.2 µg/mL] vs 1.8 µg/mL [IQR, 0.2-5.4 µg/mL]; *P* = .004) and lower levels of SLPI (median, 7.7 µg/mL [IQR, 1.3-11.1 µg/mL] vs 0.5

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TABLE 1] Patient Demographics, Clinical Characteristics, and Prior Treatments of Patients Stratified in Each Cluster

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Variable	All Patients (n = 128)	Cluster 1 (n = 33)	Cluster 2 (n = 74)	Cluster 3 (n = 21)	*P* Value
Age, y	69 ± 10	70 ± 3	70 ± 1	72 ± 2	.7
Female sex	72 (56.3)	18 (54.5)	42 (56.8)	12 (57.1)	.9
Smoking status					
Never	76 (59.4)	19 (57.6)	43 (58.1)	14 (66.7)	.1
Former	30 (23.4)	5 (15.2)	18 (24.3)	7 (33.3)	...
Current	22 (17.2)	9 (27.3)	13 (17.6)	0 (0)	...
Comorbidities					
Cardiovascular disease	29 (22.7)	6 (18.2)	21 (28.4)	2 (9.5)	.1
Diabetes mellitus	12 (9.4)	6 (18.2)	5 (6.8)	1 (4.8)	.1
Stroke	8 (6.3)	2 (6.1)	5 (6.8)	1 (4.8)	.9
COPD	23 (21)	7 (21.9)	12 (20.3)	4 (21.1)	1
Treatment					
LABA	76 (59.4)	24 (72.7)	35 (47.3)	17 (81.0)	.004
LAMA	42 (32.8)	13 (39.4)	22 (29.7)	7 (33.3)	.6
Inhaled corticosteroids	55 (43)	13 (39.4)	27 (36.5)	15 (71.4)	.01
Inhaled antibiotics	6 (4.7)	2 (6.1)	2 (2.7)	2 (9.5)	.4
Cause					
Idiopathic	58 (45.3)	11 (33.3)	37 (50)	10 (47.6)	.3
After infection	21 (16.4)	6 (18.2)	11 (14.9)	4 (19)	...
After TB	12 (9.4)	6 (18.2)	6 (8.1)	0 (0)	...
Others	37 (28.9)	10 (30.3)	20 (27)	7 (33.3)	...
Past history of pertussis	7 (5.5)	2 (12.5)	1 (1.9)	4 (26.7)	.008
Exacerbations previous 1 y					
0	23 (18)	6 (18.2)	13 (17.6)	4 (19)	.9
1-2	41 (32)	11 (33.3)	25 (33.8)	5 (23.8)	...
3 or more	64 (50)	16 (48.5)	36 (48.6)	12 (57.1)	...

Data are presented as No. (%) or mean ± SD, unless otherwise indicated. LABA = long-acting β agonist; LAMA = long-acting muscarinic antagonist.

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µg/mL [IQR, 0.2-3.1 µg/mL]; *P* = .008). No significant differences were observed in lactoferrin (median, 113.2 µg/mL [IQR, 45.9-240.4 µg/mL] vs 59.4 µg/mL [IQR, 13.8-168.8 µg/mL], respectively; *P* = .2) or lysozyme (68.9 µg/mL [IQR, 39.3-104.7 µg/mL] vs 80.5 µg/mL [IQR, 57-121.3 µg/mL], respectively; *P* = .5) levels.

Cluster Classification

We then studied the relationship between AMPs to apply clustering strategies. We found strong correlations only between lactoferrin and lysozyme (ρ = 0.468; *P* < .0001) and LL-37 (ρ = 0.698; *P* < .0001). After confirming that LL-37 and SLPI did not show strong multicollinearity, we used them as clustering variables. Table 1 also shows that cluster 3 included the highest percentage of patients taking inhaled corticosteroids (*P* = .01). However, AMP levels were comparable between treated and nontreated patients with inhaled corticosteroids in each cluster (data not shown). Cluster 3 also included the highest percentage of patients taking long-acting β agonists (*P* = .004) and of patients with a history of pertussis (*P* = .008).

Airway AMP Levels

The sputum profile of AMPs in each cluster is shown in Figure 1A. All these AMPs were significantly different between clusters (*P* < .0001). We found that cluster 1 was comparable with control participants. Cluster 2 showed lower lysozyme and SLPI levels than control participants, whereas cluster 3 showed higher LL-37 and lactoferrin levels and lower SLPI levels than control participants.

Airway Infection and Disease Severity

The percentage of airway infection was significantly different among clusters: 42.4% of infected patients in

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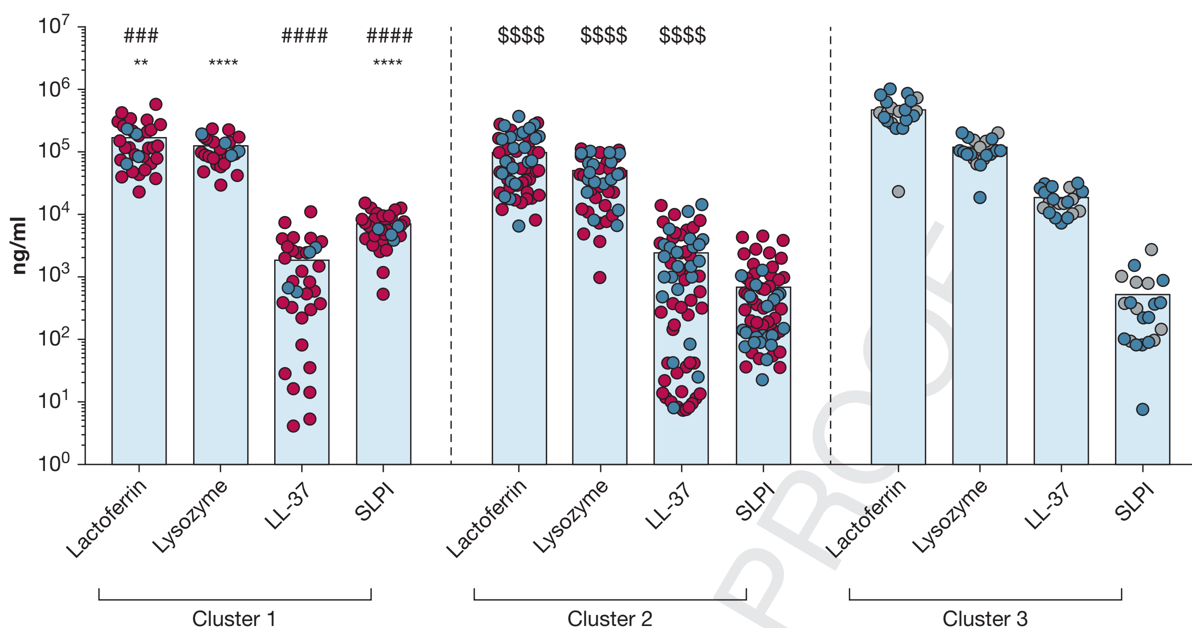

Figure 1 – *Graph showing the profile of the airway levels of antimicrobial peptides (lactoferrin, lysozyme, LL-37, and SLPI levels) in each cluster of patients (n = 128). Red circles showed the position of patients infected by P. aeruginosa. The adjusted P values are obtained by Bonferroni or Dunn test correction, according to their normal distribution. SLPI = secretory leukocyte protease inhibitor. *P < .05; **P < .01; ***P < .001; ****P < .0001. *cluster 1 and cluster 2; #cluster 1 and cluster 3; $cluster 2 and cluster 3.*

cluster 1, 67.6% of patients in cluster 2, and 85.7% of patients in cluster 3 ($P = .005$). *P. aeruginosa* infection was present in 12.1%, 31.1%, and 52.4% of patients, respectively ($P = .006$) (Fig 2A). Cluster 3 patients showed the highest severity assessed by Bronchiectasis Severity Index ($P = .01$) and FEV_1, Age, Chronic

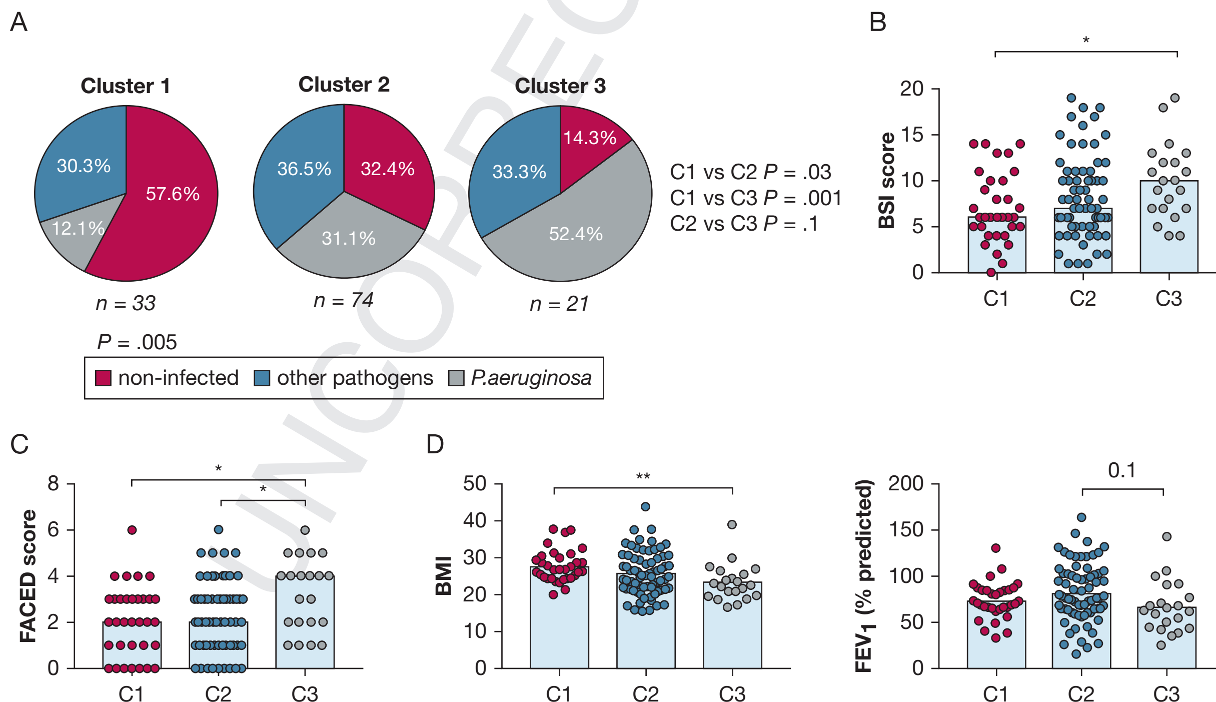

Figure 2 – *A-D, Airway infection and baseline clinical parameters in each cluster of patients. A, Percentage of airway infection. P value obtained by* χ^2 *test. B, Bronchiectasis Severity Index score. C, FACED score. D, Parameters included in these scores such as BMI and* FEV_1 *percentage predicted (n = 128). The adjusted P values are obtained by Bonferroni or Dunn test correction, according to their normal distribution. C = cluster; FACED =* FEV_1*, Age, Chronic Colonization, Extension, and Dyspnea.*

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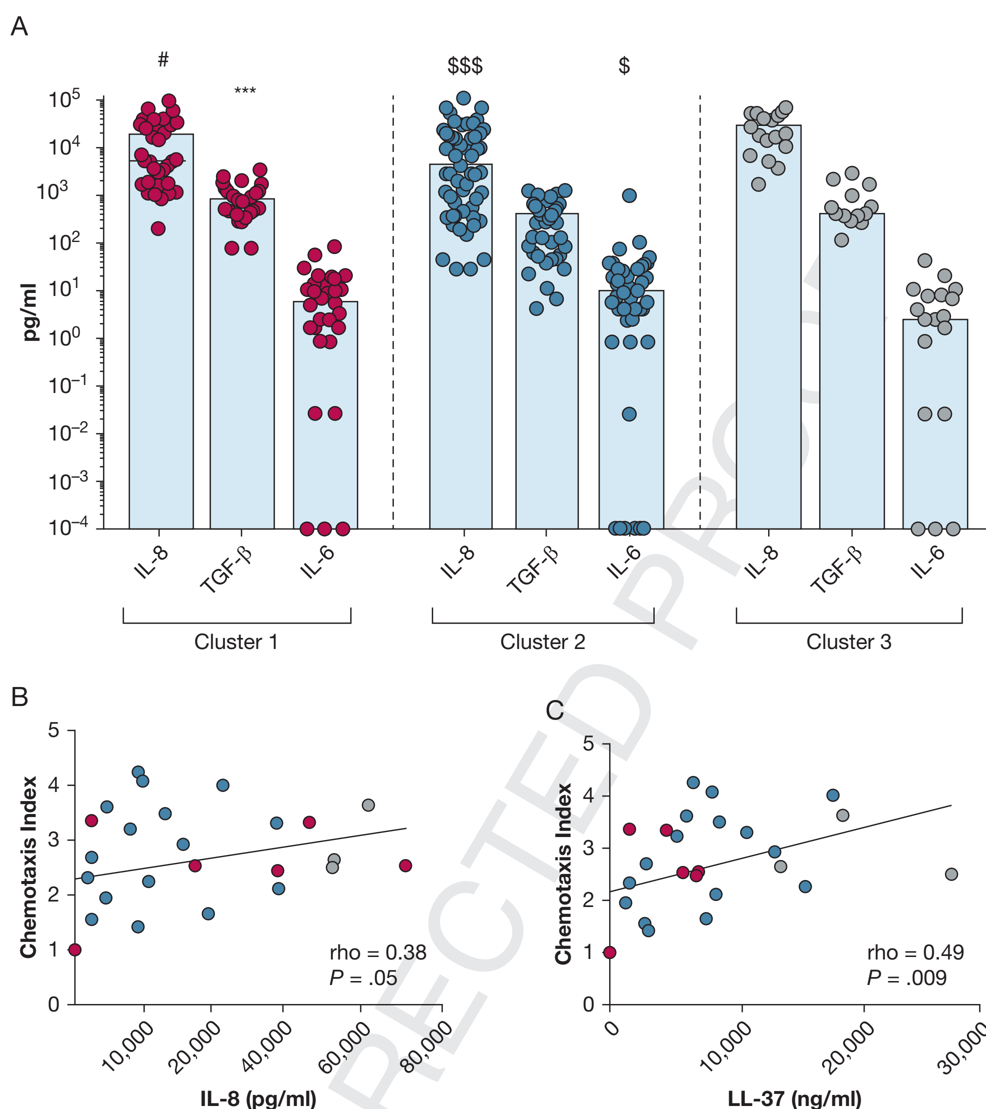

Figure 3 – A-C, Inflammatory mediators in each cluster of patients. A, Profile of IL-8, tumor growth factor β (TGF-β), and IL-6 levels in each cluster (n = 128). The adjusted P values are obtained by Dunn's test correction. B-C, Relationship between chemotaxis of healthy blood neutrophils cultured in sputum supernatants (n = 25) and the content of IL-8 (B) and (C) LL-37 sputum levels. Correlations were analyzed using the Spearman test. ***P < .001. *cluster 1 and cluster 2; #cluster 1 and cluster 3; $cluster 2 and cluster 3.

Colonization, Extension, and Dyspnea score ($P = .02$) and the lowest values of BMI ($P = .01$). This finding was not influenced by gender or age. Cluster 3 also showed the lowest value of FEV_1 percentage predicted, although only a trend was observed between clusters ($P = .07$) (Fig 2B-D).

Inflammation

Cluster 3 patients showed the highest IL-8 ($P = .0003$) and lower IL-6 levels than cluster 2 patients ($P = .01$). Cluster 2 patients showed lower TGF-β levels than cluster 1 patients ($P = .0002$) (Fig 3A). Because bronchiectasis is characterized by neutrophilic inflammation,[4] we tested the capacity of sputum supernatants from bronchiectasis patients to induce the chemotaxis of healthy blood neutrophils. Although we did not observe statistically significant differences among clusters, we did observe a positive correlation between the chemotaxis index and the sputum content of IL-8 ($\rho = 0.38$; $P = .05$) and LL-37 ($\rho = 0.49$; $P = .009$) (Fig 3B, 3C).

Tissue Remodeling and Airway Damage

Cluster 3 patients showed the highest levels of GAGs ($P < .0001$), matrix metallopeptidase 9 ($P = .004$), and neutrophil elastase activity ($P = .005$) (Fig 4A-C). We also observed that cluster 3 patients showed the highest levels of total DNA and bacterial DNA ($P < .0001$) (Fig 4D). The overall analysis revealed a distinct profile of relationships

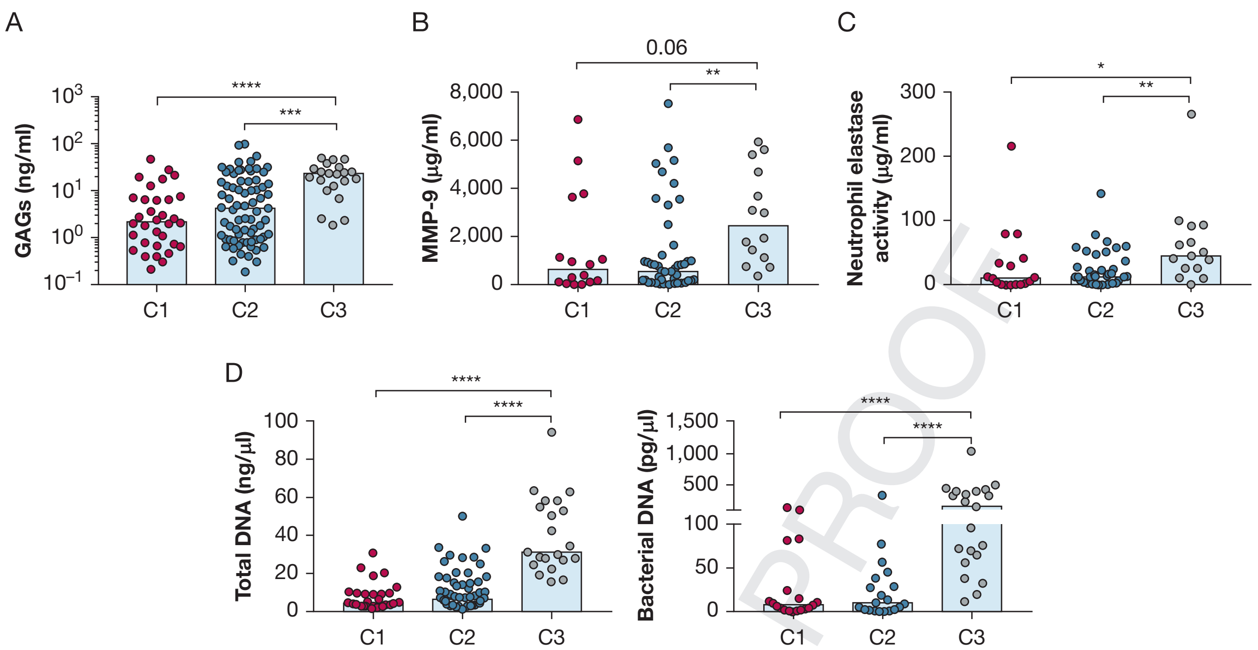

Figure 4 – A-D, Tissue remodeling and airway epithelial damage in each cluster of patients. A, Sulphated GAG levels (n = 128). B, MMP-9 levels (n = 81). C, Activity of neutrophil elastase (n = 78). D, Total and bacterial DNA levels (n = 128 and n = 60, respectively). The adjusted P values are obtained by Dunn's test correction. C = cluster; GAG = glycosaminoglycan; MMP-9 = matrix metallopeptidase 9. *P < .05; **P < .01; ***P < .001; ****P < .0001.

among AMPs, inflammatory mediators, and markers of tissue remodeling and damage in each cluster (e-Fig 1).

Longitudinal Outcomes

Ninety-eight patients experienced exacerbation during the 1-year follow-up. Of them, 24 patients (24.5%) experienced severe exacerbations. In cluster 3, 66.7% of patients experienced three or more total exacerbations during follow-up, compared with 37.8% in cluster 2 and 24.2% in cluster 1 ($P = .03$) (Fig 5A). Cluster 3 patients also experienced more severe exacerbations compared with cluster 2 and cluster 1 patients (0.8 ± 1.5 vs 0.2 ± 0.6 vs 0.1 ± 0.3, respectively; $P = .006$) (Fig 5B). Cluster 3 patients showed a shorter time to first exacerbation compared with cluster 1 patients (hazard ratio, 2.1; 95% CI, 1.0-4.2; $P = .02$) and a tendency toward a shorter time to first exacerbation compared with cluster 2 patients (hazard ratio, 1.4; 95% CI, 0.8-2.5; $P = .2$) (Fig 5C). Interestingly, among the patients with and without *P. aeruginosa* infection was a similar percentage of patients in cluster 3 who were hospitalized (41% and 44%, respectively) who had experienced 3 or more exacerbations (both 66.6%) and who experienced an exacerbation in the next 5 months (83% and 77%, respectively). We also observed that cluster 2 patients experienced exacerbation earlier than cluster 1 patients (hazard ratio, 1.7; 95% CI, 1.1-2.6; $P = .04$) (Fig 5C). Interestingly, the comparison of clinical outcomes among clusters revealed us that none of the cluster 1 patients, but 50% of cluster 2 patients and 41% of cluster 3 patients, with *P. aeruginosa* infection were hospitalized.

Discussion

In this study, we applied a new strategy combining airway AMPs to identify three biological clusters associated with distinct profiles of airway inflammation, tissue remodeling, and tissue damage. Furthermore, the three clusters showed distinct degrees of past, current, and future clinical parameters. This tool helped us to identify a cluster (cluster 3) with the highest severity and the highest risk of future exacerbations characterized by a deregulated local innate immune response and increased tissue remodeling and damage. We also identified two clusters (clusters 1 and 2) with low tissue remodeling and damage that could explain their severity.

Our cluster strategy was based on LL-37 and SLPI because of their independence and their marked association with Bronchiectasis Severity Index, airway infection, and risk of exacerbation.[10] The three clusters presented different airway AMP profiles. Cluster 3, with the highest levels of LL-37 and lactoferrin and the lowest levels of SLPI, were the patients with the highest severity. Inversely, cluster 1, which showed an airway AMP profile similar to control participants, were the mildest patients. Finally, cluster 2, with low SLPI and lysozyme levels, showed moderate severity. It should be

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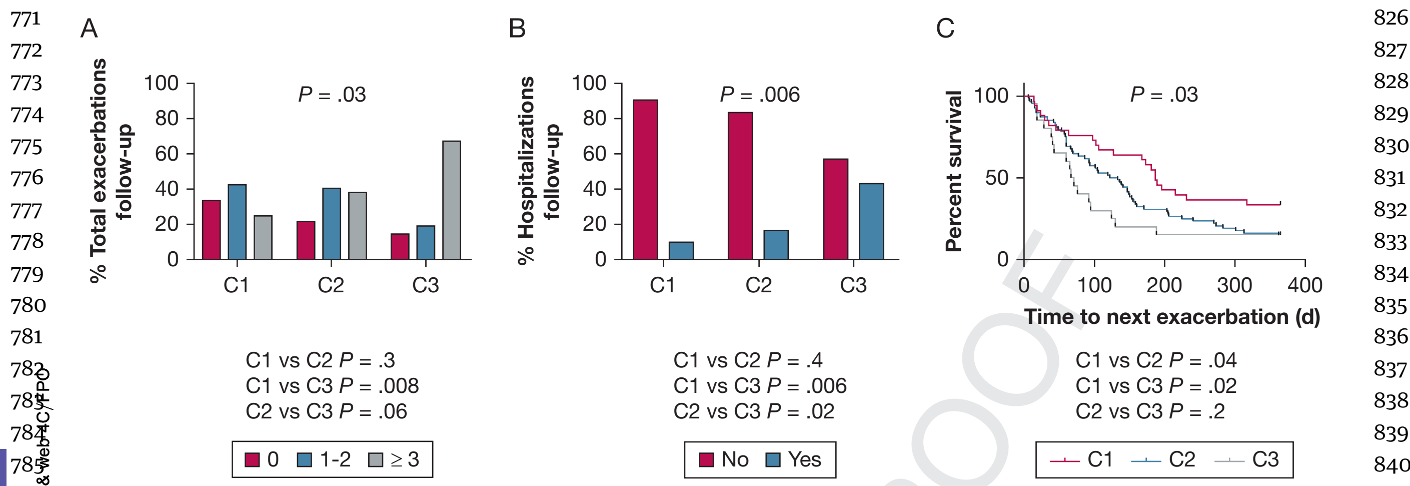

Figure 5 – A-C, Relationship between clusters and clinical outcomes. A, Percentage of the total exacerbations during 1 year of follow-up (n = 128). B, Percentage of severe exacerbations that required hospitalizations during 1 year of follow-up (n = 128). C, Percentage of patients free of exacerbation in each C (n = 128). The log-rank (Mantel-Cox) test was used. C = cluster.

mentioned that our control airway AMP levels are comparable with those of other reports.[11,24] Compared with other work,[17-19] we obtained clusters based on AMPs, rather than on extensive panels of sputum inflammatory mediators, because AMPs mainly are neutrophil proteins and are closely related to airway infection.

We found that airway AMP profiles in each cluster were associated distinctly with airway infection. Interestingly, cluster 3 patients, with high AMP levels, were expected to have a greater protection against infection. However, they showed the highest percentage of airway infection. This apparent contradiction could be explained by Cole's vicious cycle hypothesis,[25] suggesting that chronic inflammation contributes to the persistence of bacteria, which leads to greater inflammation. In fact, cluster 3 patients frequently were infected with *P. aeruginosa*, which is linked to great inflammation, severity, and poor outcomes.[26] We also found that patients infected by *P. aeruginosa* showed a heterogeneous airway immune profile. Therefore, both AMPs and *P. aeruginosa* should be explored together in future extensive cohorts. Although cluster 1 and 2 patients demonstrated lower inflammation and better outcomes than cluster 3 patients, 12% and 31% of patients, respectively, were infected with *P. aeruginosa*. This finding has several possible explanations. One is a lower bacterial load in clusters 1 and 2 than in cluster 3.[27] Although data for the quantitative bacterial load are not available, we found that cluster 3 had the highest sputum bacterial DNA, which is in line with this hypothesis. Another possibility is that clusters are associated with different *P. aeruginosa* strains in terms of virulence factors, biofilm production, and antimicrobial resistance that favor their persistence in the lungs.[28-30] Thus, we speculate that cluster 3 could be the cluster with the most virulent and resistant *P. aeruginosa* strains. Regarding infections, we did not observe any association between clusters and causes of infection. However, it should be mentioned that only 9.4% of patients had recovered from TB, and a more extensive cohort is needed to reach conclusions about the impact of causes in our cluster classification.

We found that LL-37 levels are correlated with in vitro neutrophil migration toward sputum, confirming that this AMP has additional functions. LL-37 also is associated with endotoxin binding, wound healing, release of histamine and leukotriene B4, and modulation of dendritic cell function.[31] At physiologic concentrations, LL-37 properties are beneficial for the host defense, but at higher concentrations, LL-37 has a cytotoxic effect on leukocytes and epithelial cells.[32,33] The high levels of total DNA in cluster 3 patients suggest the death of neutrophils and epithelial cells resulting from cytotoxic LL-37 levels. It is reported that sputum[34] and blood[35] neutrophils from bronchiectasis patients showed delayed apoptosis. However, the sputum soluble factors reflect the consequences of the presence of neutrophils in airways. We determined IL-8 levels to be an indirect measure of the presence of neutrophils, and cluster 3 showed the highest IL-8 levels. We did not find differences in IL-8 and total and bacterial DNA between clusters 1 and 2, suggesting comparable numbers of sputum neutrophils.

We found different GAGs levels in the sputum from bronchiectasis patients. Cluster 3 showed the highest airway sulphated GAGs levels, followed by cluster 2, suggesting an excessive tissue remodeling characteristic

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of excessive inflammation and an imbalance between pulmonary proteases and antiproteases.[36] Cluster 3 showed the highest matrix metallopeptidase 9 levels and neutrophil elastase activity. They contribute to the damage of airway epithelium through the breakdown of GAGs[37] and render the airways more susceptible to infection.[25] The analysis of the relationship between the immune parameters determined revealed that almost all the relationships observed in clusters 1 and 2 were absent in cluster 3. This suggests that the poor outcomes in cluster 3 could be linked to deregulated airway immune responses. We also observed that cluster 3 included a high percentage of patients treated with inhaled corticosteroids. Although we cannot infer whether this treatment is the cause or the consequence, studies have reported the inhibitory effects of inhaled corticosteroids on AMPs.[38,39] Other known regulatory factors of AMP production are vitamin D levels,[40] which were not evaluated in this study, and smoking status,[41] which did not show any influence in the clustering of the cohort. Therefore, the severity of bronchiectasis could be a combination of factors, including inflammation, remodeling, and deregulation of certain AMPs. Although we did not find significant differences in the LL-37 levels between clusters 1 and 2 or in the SLPI levels between clusters 2 and 3, these clusters were significantly different in inflammatory mediators, tissue remodeling and damage, severity, and clinical outcomes. These findings suggest that the AMP profile is relevant to stratify the bronchiectasis patients with distinctive clinical parameters and future exacerbations.

Our study has some limitations. First, we had no consecutive samples during the follow-up. Therefore, we could not analyze the long-term stability of these clusters. Second, we are aware of the risk of spurious associations resulting from multiple statistical comparisons. However, we focused the cluster analysis on two independent markers (LL-37 and SLPI) clearly associated with clinical parameters at baseline. Finally, airway infection was determined by conventional microbiological cultures instead of molecular diagnosis, which may explore better the characteristics of airway infection in each cluster.

Interpretation

Our cluster strategy identifies three clusters of bronchiectasis patients with distinct profiles of AMPs, inflammation, and lung remodeling and damage. These profiles are translated into distinct and gradual clinical phenotypes in terms of airway infection, disease severity, and outcomes, strengthening the validity of these biological clusters. However, it is important to validate these clusters externally. Furthermore, our work gives relevance to the existence of distinct relationships among airway immune response mediators in each biological cluster. Therefore, our work highlights the importance of identifying patients with distinct grades of severity based on the airway immune profile to improve therapies to restore pulmonary immune homeostasis.

Acknowledgments

Author's contributions: L. P. and S. V. take full responsibility for the content of the manuscript, including the data analysis. L. P. and E. C. designed the study and performed experimental work, data collection, data interpretation, and statistical analysis, including writing of the final manuscript. G. S.-C., J. D. C., and S. A. carried out patient recruitment, sample collection, clinical data collection, and data analysis. O. S. and S. V. designed the study, obtained study funding, interpreted results, carried out data analysis, and wrote the final manuscript. All authors reviewed and approved the final draft of the manuscript.

Financial/nonfinancial disclosures: The authors have reported to *CHEST* the following: J. D. C. has received research grants from GlaxoSmithKline, Boehringer-Ingelheim, AstraZeneca, Gilead Sciences, Zambon, and Insmed and has received personal fees from Boehringer-Ingelheim, AstraZeneca, GlaxoSmithKline, Chiesi, Novartis, Insmed, and Zambon. S. A. has received research grants from Chiesi, Insmed, and Fisher & Paykel and has received personal fees from Bayer HealthCare, Grifols, AstraZeneca, Zambon, Chiesi, Insmed, GlaxoSmithKline, Menarini, and ZetaCube Srl. None declared (L. P., E. C., G. S.-C., O. S., S. V.).

Additional information: The e-Figure and e-Tables can be found in the Supplemental Materials section of the online article.

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