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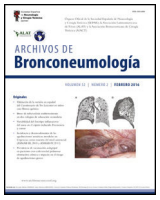

Original Article

Clinical and Lung Microbiome Impact of Chronic Versus Intermittent *Pseudomonas aeruginosa* Infection in Bronchiectasis

Laia Fernández-Barat[a,b,c,*], Ruben López-Aladid[a,b,c], Victoria Alcaraz-Serrano[a,b,c], Nil Vázquez[a,b,c], Leticia Bueno-Freire[a,b,c], Roque Pastor-Ibañez[a,b,c], Lena Lingren[f,g], Héctor Sanz-Fraile[b], Patricia Oscanoa[a,b,c], Ana Motos[a,b,c], Roberto Cabrera[a,b,c], Jordi Vila[e], Daniel Martínez[d], Jordi Otero[b], Ramon Farré[b], Niels Høiby[f,g], Antoni Torres[a,b,c]

[a] *CELLEX Research Laboratories, CibeRes (Centro de Investigación Biomédica en Red de Enfermedades Respiratorias, 06/06/0028), Institut d'Investigacions Biomèdiques August Pi i Sunyer (IDIBAPS), Barcelona, Spain*
[b] *School of Medicine, Department of Medicine & Department of Biophysics and Bioengineering, University of Barcelona, Spain*
[c] *Pulmonology Department, Hospital Clínic, Barcelona, Spain*
[d] *Pathology Department, Hospital Clínic, Barcelona, Spain*
[e] *Microbiology Department, Hospital Clínic, CRESIB ISglobal, Barcelona, Spain*
[f] *Department of Clinical Microbiology of Rigshospitalet, Copenhagen, Denmark*
[g] *Institute of Immunology and Microbiology, University of Copenhagen, Copenhagen, Denmark*

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ABSTRACT

Background: In patients with non-cystic fibrosis bronchiectasis (BE) *Pseudomonas aeruginosa* (PA) has been recently associated with low rather than high number of exacerbations without distinguishing chronic versus intermittent infection. The aim of our study was to determine whether the intermittent or chronic stage of *P. aeruginosa* (PA) infection is associated with the rate of exacerbations, quality of life and respiratory microbiome biodiversity after a one-year follow-up.
Methods: We conducted a longitudinal study, with 1-year follow-up, in patients with BE intermittently or chronically infected by PA involving sequential (3-monthly) measurements of microbiological (cultures, PA load, phenotype and biofilms presence) immunological (Serum IgGs against *P. aeruginosa* were measured by ELISA immunoassay) and clinical variables (Quality-of-Life and the number exacerbations). Additionaly, 16S sequencing was performed on a MiSeq Platform and compared between chronically infected patients with the mucoid PA versus intermittently infected patients with the non-mucoid PA.
Results: We collected 235 sputa and 262 serum samples from 80 BE patients, 61 with chronic and 19 with intermittent PA infection. Chronically compared to intermittently.
Presented reduced quality of life but less hospitalized exacerbations after 1-year follow-up. Chronically infected patients presented reduced sputum biodiversity and higher systemic IgGs against *P. aeruginosa* levels that were associated to decreased number of hospitalized exacerbations.
Conclusions: The assessment of Chronic versus intermittent *P. aeruginosa* infection has clinical implications such as quality of life, rate of hospitalized exacerbations and lung microbiome biodiversity. The distinction of these two phenotypes is easy to perform in clinical practice.
Trial registration: XXXXXX.

Introduction

Chronic infection by *Pseudomonas aeruginosa* (PA) is reported in 12–27% of patients with non-cystic fibrosis (non-CF) bronchiectasis (BE). PA in BE compared to other pathogens entails a worse prognosis: a 3-fold increase in mortality risk, and up to a 7-fold increase in the risk of hospital admission.[1–3]

Intermittent infection by PA is often the first step towards the onset of chronic airway infection, which carries poor prognostic outcomes.[3–5] Underdiagnosis of PA infection at early stages can therefore have significant consequences in patients with an underlying chronic respiratory disease.[6] Unless eradicated, non-mucoid PA can shift to a mucoid PA phenotype. Growing in aggregates and

* Corresponding author.
E-mail address: lfernan1@recerca.clinic.cat (L. Fernández-Barat).

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the alginate produced by the mucoid[7,8] not only confers protection towards antimicrobials and immune host defences, but becomes a foci for systemically spreading infections and leads to the emergence of multidrug-resistant strains difficult to culture.[9–13]

Unfortunately, the possibility of false-negative results in Standard-of-Care Tests (SOCT) is well documented for biofilm infections.[9,14–16] In 2015 the European Society of Clinical Microbiology and Infectious Diseases (ESCMID) issued its first guidelines with recommendations to improve the diagnosis and treatment of various biofilm-related infections not including BE. Briefly, these recommendations included the confirmation of bacterial aggregates in the sample by microscopy images and the use of sonication to release microorganisms from biofilms aggregates before culturing.[15] ESCMID methods also higlighted the high sensitivity (93–97%) and specificity (83–89%) of systemic IgGs against *P. aeruginosa* (antiPA-IgGs) to confirm chronic infection in patients with cystic fibrosis (CF).[9,14]

Over the course of a 3-year longitudinal study, we aimed to compare the number of exacerbations, quality of life and microbiome biodiversity between patients with intermittent or chronic PA infection. Additionally, to improve PA diagnosis and characterization of the mucoid versus non-mucoid phenotype we compared ESCMID methods for biofilms with SOCT in all samples.

Methods

Study Design and Participants

This ambispective longitudinal cohort study (XXXX) of patients with BE was conducted at a tertiary care hospital, and research laboratories. We included adult patients (>18 years) diagnosed with BE of any aetiology by high-resolution computerized tomography (HRCT) of the chest and with at least one previous PA isolation in sputum 1 year before inclusion. Patients were enrolled from May 2017 to November 2019. Written informed consent was obtained from all patients and the study was approved by the Internal Review Board of the Hospital (registry number XXX/2018/0236).

Definitions

Exacerbation was defined as a deterioration in three or more of the following symptoms: cough, sputum volume and/or consistency, sputum purulence, dyspnoea and/or exercise tolerance, fatigue or general malaise, and hemoptysis.[17] *Chronic PA infection* was defined as two or more isolates separated by 3 months in a single year according to published guidelines.[2] We included patients who met the definition of chronic infection at inclusion based on their clinical histories including microbiology (retrospective) and/or those whose cultures (by SOCT and/or ESCMID methods) at follow-up accomplished the definition of chronic infection (prospective). Patients who neither retrospectively nor prospectively did not met the definition of chronic infection were allocated in the intermittently infected group.

Procedures

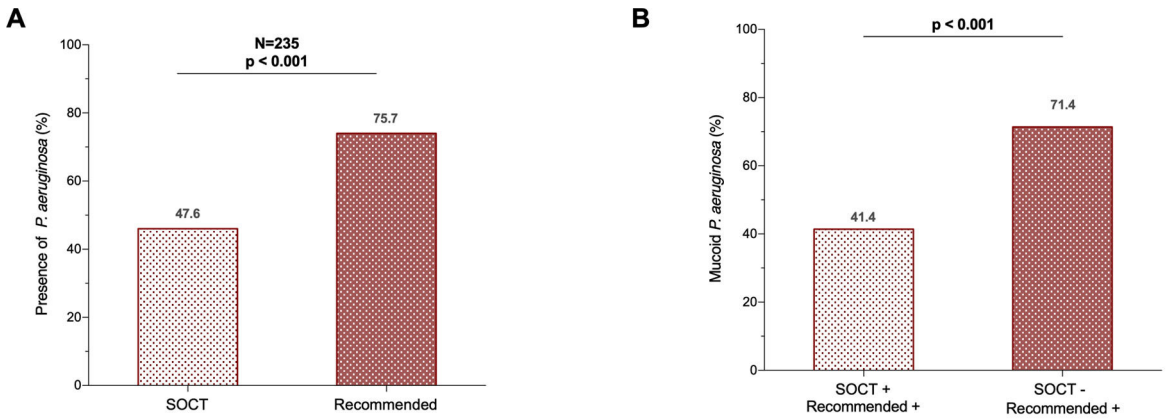
Demographics and clinical data were recorded at baseline. Patients attended the clinic once every 3 months during the stable phase throughout the study period. At each visit: (i) spontaneous sputum and blood samples were taken (also at exacerbations within the first 48 h); (ii) lung function was assessed; and (ii) the Quality-of-Life Bronchiectasis questionnaire was completed. Patients were asked to call a 24/7 phone line if they experienced any of the symptoms of an exacerbation. The number of mild and hospitalized exacerbations was recorded prospectively and also 3 years retrospectively based on the clinical history (Table 1).

Each sputum sample was collected to assess the following: (1) SOCT and Gram Staining, (2) ESCMID recommended cultures (Recommended) for biofilm infections and Gram staining for detecting alginate presence, (3) Fluorescence in situ hybridization (FISH) + Confocal microscopy, (4) Microbiome analysis, and (5) Rheological properties of mucus. Only sputum samples of Murray-Washington classification degrees IV, V or VI, which are considered of good quality, were included in the analyses.[18] SOCT and ESCMID recommended cultures are described in Fig. 1C and in the online data supplement.[10,14] *P. aeruginosa* in sputum smears was identified by FISH using the PA-specific PNA probe32 (AdvanDx, USA). Images of PA biofilms were obtained using a confocal microscope equipped with appropriate filters, as described elsewhere.[10] For

Table 1
Characteristics of BE Patients With Intermittent vs. Chronic *P. aeruginosa* Infection.

Variable	Intermittent PA *N* = 19	Chronic PA *N* = 61	*p*-Value
Gender, M/F	9 (47)/10 (53)	24 (39)/37 (61)	0.599
Age, years	77 [68–81]	76 [66–83]	0.830
Mean PA load (Log CFU/mL)	3.90 ± 2.25	5.44 ± 1.03	**<0.001**
PA phenotype			**<0.001**
Non-Muc	17 (89)	25 (41)	
Muc	2 (11)	36 (59)	
Anti-P.aeruginosa IgG	3.25 [0.00–14.53]	20.23 [9.89–38.33]	**<0.001**
Alginate presence			**0.006**
No	14 (74)	30 (49)	
Yes	1 (5)	25 (41)	
Aetiology			0.780
Post-infectious	9 (47)	24 (39)	
Idiopathic	4 (21)	15 (25)	
COPD	4 (21)	8 (13)	
Others	2 (11)	14 (23)	
FEV_1(%) predicted	63.90 ± 26.70	62.70 ± 18.53	0.828
BSI	11.00[8.00–13.00]	11.50[8.00–15.00]	0.423
Mild	1 (5)	2 (4)	
Moderate	4 (21)	12 (24)	
Severe	14 (74)	37 (73)	
FACED	4.00 [3.00–4.00]	4.00 [3.00–5.00]	0.241
Mild	4 (21)	8 (15)	
Moderate	11 (58)	22 (42)	
Severe	4 (21)	22 (42)	
Chronic treatment			
Oral ATB	6 (30)	19 (32)	0.889
Inhaled ATB	5 (25)	16 (27)	0.883
Corticosteroids	12 (60)	41 (70)	0.435
Bronchodilators	17 (85)	51 (85)	1.000
Previous exacerbations			
Mild	5.00 [0.00–8.00]	5.00 [2.50–7.00]	0.968
Hospitalized	1.00 [0.00–3.00]	0.00 [0.00–2.50]	0.169

Numbers are expressed in *n* (%). Values are reported as mean ± SD or median [IQR]. Percentages calculated on non-missing data. *Abbreviations*: PA: *Pseudomonas aeruginosa*; M: male; F: female; COPD: chronic obstructive pulmonary disease; FEV_1: forced expiratory volume in the first second; BSI: Bronchiectasis Severity Index; FACED: (F: FEV_1 in % of predicted with a cut-off point of 50%; A: age with cut-off point age 70; C: presence or absence of chronic bronchial colonization/infection by PA; E: radiological extension by number of pulmonary lobes affected in CT scan, dichotomized as <2 vs. >2); D: dyspnoea, measured using the modified Medical Research Council scale, dichotomized as 0–II vs. III–IV). Chronic treatment was assessed retrospectively based on clinical history. Patients newly diagnosed with chronic PA during the three-monthly visits were allocated to the chronic group. Higher PA load, higher frequency of mucoid phenotype, higher levels of AntiPA-IgG (IgG against *P. aeruginosa*) and higher presence of alginate in Gram staining smears were significantly found in patients with chronic compared with those that have intermittent PA infection. No differences in chronic treatment were found between groups.



C

Standard microbiology methods for respiratory samples (SOCT)	ESCMID recommended microbiology methods for respiratory biofilms (Recommended)
• Gram staining for sputum quality • Blood and chocolate agar • Incubation 24-48 h • Susceptibility testing • MALDI-TOF • Ziehl–Neelsen staining and Lowenstein plate cultures as well as Mycobacteria Growth Indicator Tube liquid cultures (If suspicion of Mycobacterium spp.)	• Sputum quality and biofilm imaging by Gram-staining • Use of DTT (1:1) • Sonication 40kHz, 5 min • Mackonkey and blood Agar • Incubation 24h-96h • Colonies Forming Units (CFU) • Discrimination of mucoid and non-mucoid morphotype • PA Biofilm imaging by FISH+CLSM • Susceptibility testing • MALDI-TOF

Fig. 1. Comparison between microbial diagnosis by Standard-of-Care Tests (SOCT) and recommended cultures for biofilm infections. (A) Differences between SOCT and recommended methods in diagnosing *P. aeruginosa (PA)* presence; (B) Differences between SOCT and recommended methods in PA mucoid phenotype; (C) Microbiology following standard or recommended methods for sputum samples. SOCT+/Recommended+: samples that tested positive for PA by both SOCT and recommended methods; SOCT−/Recommended+: samples that tested positive for PA by recommended methods but negative for PA by SOCT; Recommended+/16S+: samples that tested positive for PA by recommended and 16S methods; Recommended−/16S+: samples that tested positive for PA by recommended methods but negative for PA by SOCT. DTT: dithiothreitol, FISH: fluorescent in situ hybridization, CLSM: confocal laser scanning microscopy, MALDI-TOF: matrix-assisted laser desorption/ionization time-of-flight mass spectrometry.

all imaging studies we analyzed ≥ 2 sputum smears per patient obtained 3 months apart.

For microbiome analyses we followed methods previously reported.[19] 16S profiling was compared between chronically infected patients with the mucoid PA versus intermittently infected patients with the non-mucoid PA. Rheological properties of mucus were measured using a 35-mm serrated parallel-plate rheometer (Haake Rheostress1, ThermoFisher, MA, US) as reported elsewhere.[20] IgG against the O-Antigen of *P. aeruginosa* (Anti-PA IgG) were analyzed in serum samples using ELISA at the Department of Microbiology, Rigshospitalet, University of Copenhagen, Denmark, as described previously.[10] Lung function was evaluated using the EasyOne™ World Spirometer (NDD Medical Technologies, Zurich, Switzerland), and classified according to the American Thoracic Society/European Respiratory Society Guidelines. Differences in Health-Related Quality of Life (HRQoL) (assessed with the Quality of Life Questionnaire-Bronchiectasis (QOL-B) v3.1) and the minimal important difference (MID) scores were contrasted in both the first visit and the last visit after 1 year follow-up by infection status (intermittent vs. chronic).

Statistical Analysis

Categorical and continuous variables were reported as percentages or as mean ± standard deviation (SD) or median (IQR), depending on data distribution. Group comparisons were conducted using the Chi-squared test for categorical variables and the Student's *t*-test or Mann–Whitney *U* test for continuous variables. Paired samples were analyzed using the paired *t*-test or Wilcoxon signed-rank test. A multivariable logistic regression model was used to identify independent factors associated with chronic PA status, with results expressed as odds ratios (ORs) and 95% confidence intervals (CIs). Model performance was assessed using the Hosmer–Lemeshow test and the area under the ROC curve (AUC). Statistical significance was set at $p < 0.05$. Analyses were conducted using SPSS Statistics 25.0. Complete statistical methods are provided in the Supplemental Material.

Results

Characteristics of the Study Population

During enrolment a total of 666 BE patients were screened for PA-positive sputum. PA isolates were found in the samples of 80 of these patients, who formed our study population. Of these 80, 19 (24%) met the definition of intermittently infected and 61 (76%) were chronically infected. Of the patients with chronic infection, 34 (56%) had been colonized by PA for a median [interquartile range (IQR)] of 7.00 [3.75–12.00] years and 27 (44%) were newly diagnosed during the course of the study, being colonized for 0.5

[0.5–0.5] years (Fig. 1S). The baseline clinical characteristics of the study population are summarized in Table 1. At study inclusion, patients with intermittent or chronic infection status did not differ in terms of age, gender, aetiology of BE, predicted FEV_1%, FACED score or Bronchiectasis Severity Index (BSI), nor the number of previous exacerbations. In contrast, higher PA load, AntiPA-IgG and presence of alginate and mucoid phenotype (biofilms) were significantly associated to patients with chronic infection. In intermittently infected patients, mucoid PA was barely found (Table 1).

SOCT Versus ESCMID Recommended Cultures

Two hundred and thirty-five sputum samples of good quality, with a median of 3.00 [2.00–5.00] per patient, were analyzed using both Standard-of-Care Tests (SOCT) and ESCMID (recommended) cultures immediately after sample collection (Fig. 1C).

Overall, PA isolation was higher when using the ESCMID recommended methods than with SOCT (74% vs. 44%, $p < 0.001$). Furthermore, the ESCMID recommended methods for biofilm infections allowed detection of PA in 11 (38%) of the 29 exacerbations that gave false-negative results using SOCT, and diagnosis of 18 (66%) of the 27 chronic infections that emerged during the study period, compared to nine (33%) that would have been diagnosed using SOCT alone (Fig. 1A).

The frequency of mucoid PA detected in SOCT was significantly lower than that detected in the recommended cultures (41% vs. 71%, Fig. 1B). Mucoids compared to non-mucoids needed two days or more to growth (2.00 [2.00–4.00] vs. 2.00 [1.00–2.00], $p < 0.001$, respectively), but SOCT culture plates are typically discarded after two days. The sensitivity of ESCMID Recommended cultures and Gram staining methods was superior compared to SOCT (Fig. 3S).

In a subgroup analysis ($n = 51$) using 16S rRNA gene sequencing as a culture-independent technique control, in terms of PA isolation high and similar sensitivity was found between ESCMID recommended cultures and 16S but not between SOCT and 16S (Figs. 3S A and 3S B).

In Situ Identification of PA Biofilms

The presence of PA alginate and biofilms in sputum was associated with the mucoid PA phenotype (Fig. 4S) and chronic PA (Table 1). The presence of PA biofilms in sputum from BE patients was demonstrated by FISH and confocal laser scanning microscopy, and resembled to that of the PA biofilms shown in CF patients (Fig. 2).[10]

The Mucoid Pathobiome

Chronically infected patients with the mucoid PA had a significant reduction in lung alpha and beta biodiversity compared to intermittently infected patients with the non-mucoid PA (Fig. 3 A). Community compositional dissimilarity among Chronically infected with the mucoid (red dots) and Intermittently infected with the non-mucoid (blue dots) showed significant compositional differences between them ($R^2 = 0.21$, $p_{Adonis} = 0.001$) (Fig. 3B).

Additionally, using linear discriminant analysis effect size (LEfSe) we determined the respiratory microbiota composition of chronically infected with the mucoid and Intermittently infected with the non-mucoid. Samples with mucoid PA were dominated by *Proteobacteria* phyla, with a marked abundance of *Pseudomonas* and *Neisseria* at the genus level. In contrast, microbial patterns in the non-mucoid group were more varied, including genera such as *Actinobacteria* (*Actinomyces*, *Rothia*), *Bacteroidetes* (*Prevotella*) and *Firmicutes* (*Streptococcus*, *Veillonella*, *Lactobacillus*, *Granulicatella*) (Fig. 3C). The mucoid pathobiome of chronically infected patients

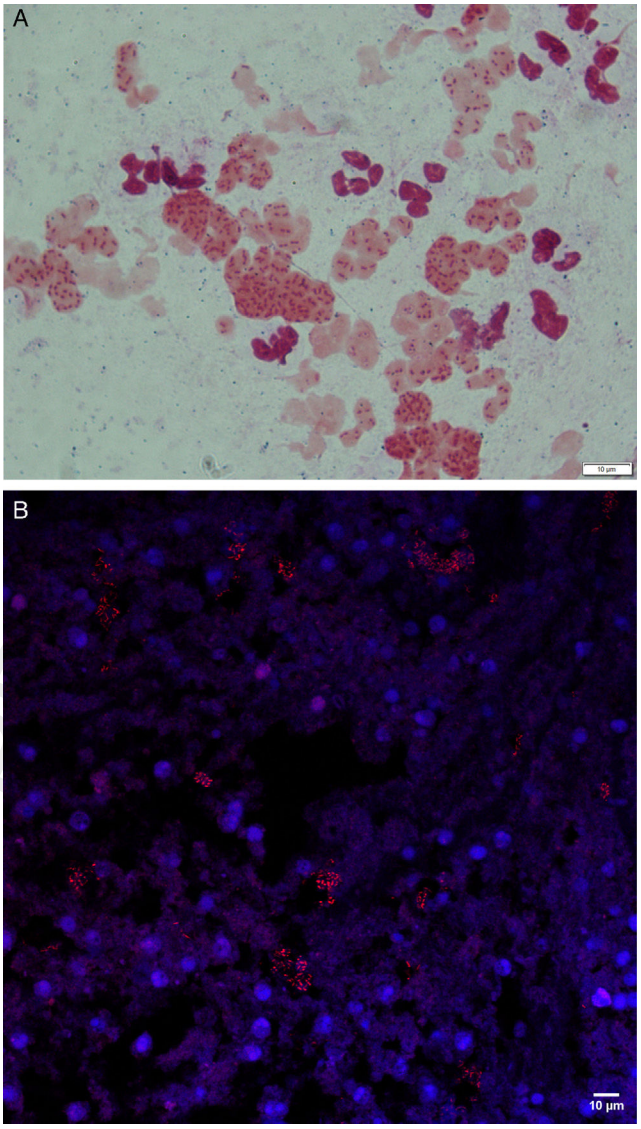


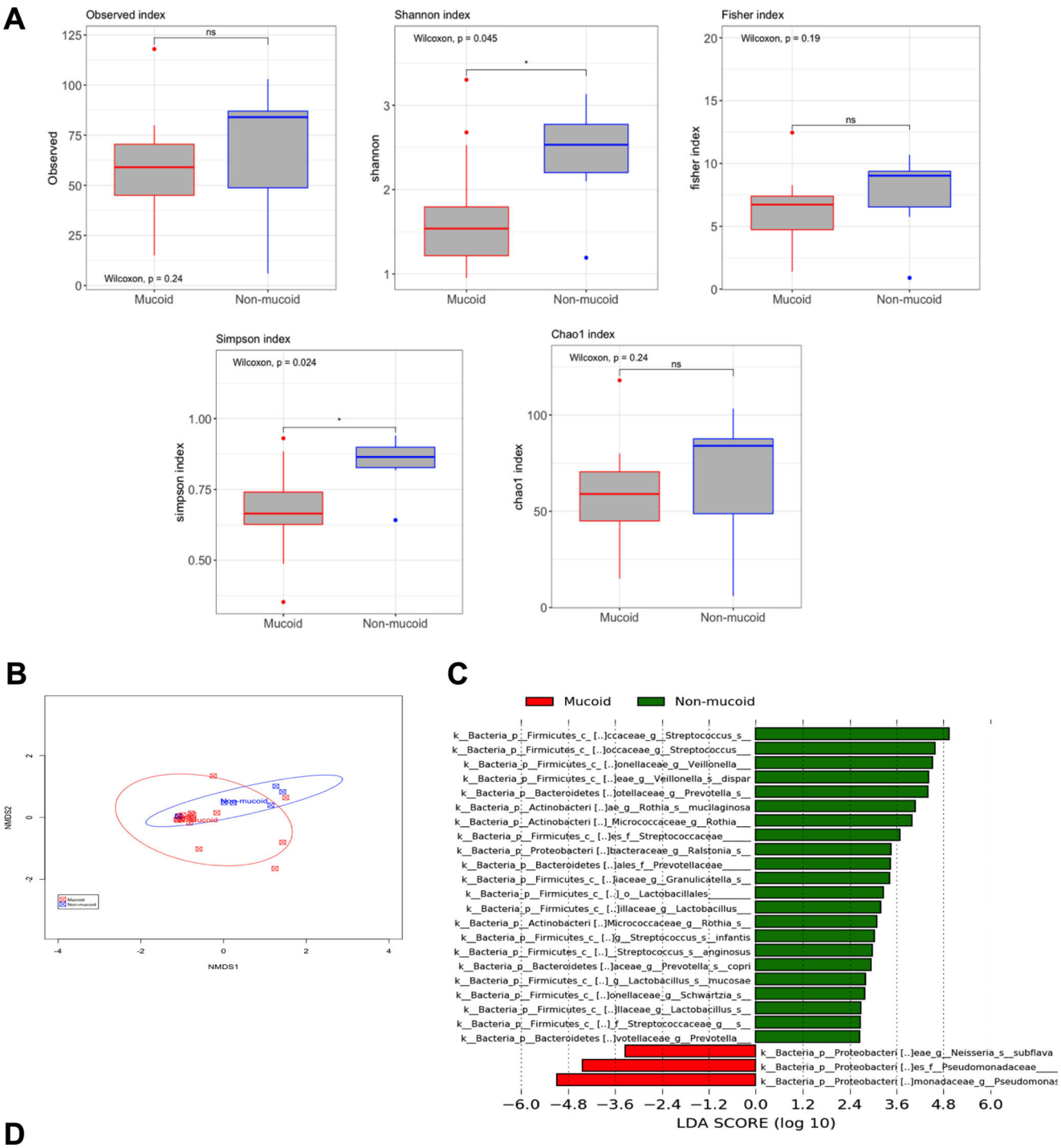
Fig. 2. Demonstration of *P. aeruginosa (PA)* biofilms by Gram staining and confocal laser scanning microscopy (CLSM) and FISH and its added diagnostic value. First demonstration of PA biofilms by Gram staining microscopy (left lung) and by CLSM + FISH in patients with non-Cystic Fibrosis bronchiectasis. *Pseudomonas aeruginosa* biofilms are tolerant of the host immune response: see polymorphonuclear leukocytes surrounding PA biofilms. The sputum in the Gram staining image (63× oil immersion objective) was obtained from a patient with bronchiectasis and chronic PA infection for 12 years (mean PA load: 5.38 LogCFU/mL, mean precipitating PA antibodies: 21.25 µ/mL and relative abundance of 42.21% of PA). In the CLSM + FISH (63× oil immersion objective) image, a specific *Pseudomonas aeruginosa* probe (red fluorescence) shows PA in biofilm aggregates and polymorphonuclear leukocytes (blue fluorescence) surrounding PA biofilms. The sputum of the CLSM + FISH picture was obtained from a patient with bronchiectasis and chronic PA infection for 12 years (mean PA load: 5.61 LogCFU/mL, mean precipitating PA antibodies: 31.86 µ/mL and relative abundance of 13.35% of PA).

was associated with higher PA load, higher systemic AntiPA-IgG and more years of chronic infection (Fig. 3D).

In terms of mucus viscoelasticity, the mucoid group ($n = 47$, 76%) presented worse viscoelastic properties at 1 rad/s than the non-mucoid group ($n = 15$, 24%) (Table 1S).

The Value of IgGs

We analyzed 262 BE patient serum samples (an average of 4.00 [2.00–4.50] independent samples per patient) collected at follow-up visits. Higher levels of IgG against the O-Antigen of



D

	Mucoid Pathobiome (No)	Mucoid Pathobiome (Yes)	P-value
PA Load (LogCFU/mL)	2.42[1.30-4.20]	5.61[4.87-6.69]	0.001
AntiPAIgG	8.76[0.71-19.08]	28.67[11.67-51.75]	0.021
Years of chronic infection	0.50[0.00-0.50]	6.00[0.50-12.00]	0.012

Fig. 3. Diversity of respiratory microbiome and linear discriminant analysis effect size (LefSE) clustered by mucoid or non-mucoid *P. aeruginosa (PA)*. (A) Alpha diversity, measured by Chao1, Fisher and Shannon's and Simpson's diversity index is plotted for patients with the mucoid phenotype/chronic PA (red) and non-mucoid phenotype/intermittent PA (blue). The line inside the boxplot represents the median. The lowest and highest values within the 1.5 interquartile range are represented by the whiskers. The dots show the outliers and individual sample values. (B) Principal coordinate analysis (PCA) based on Bray–Curtis dissimilarity. (C) The LDA finds taxa that

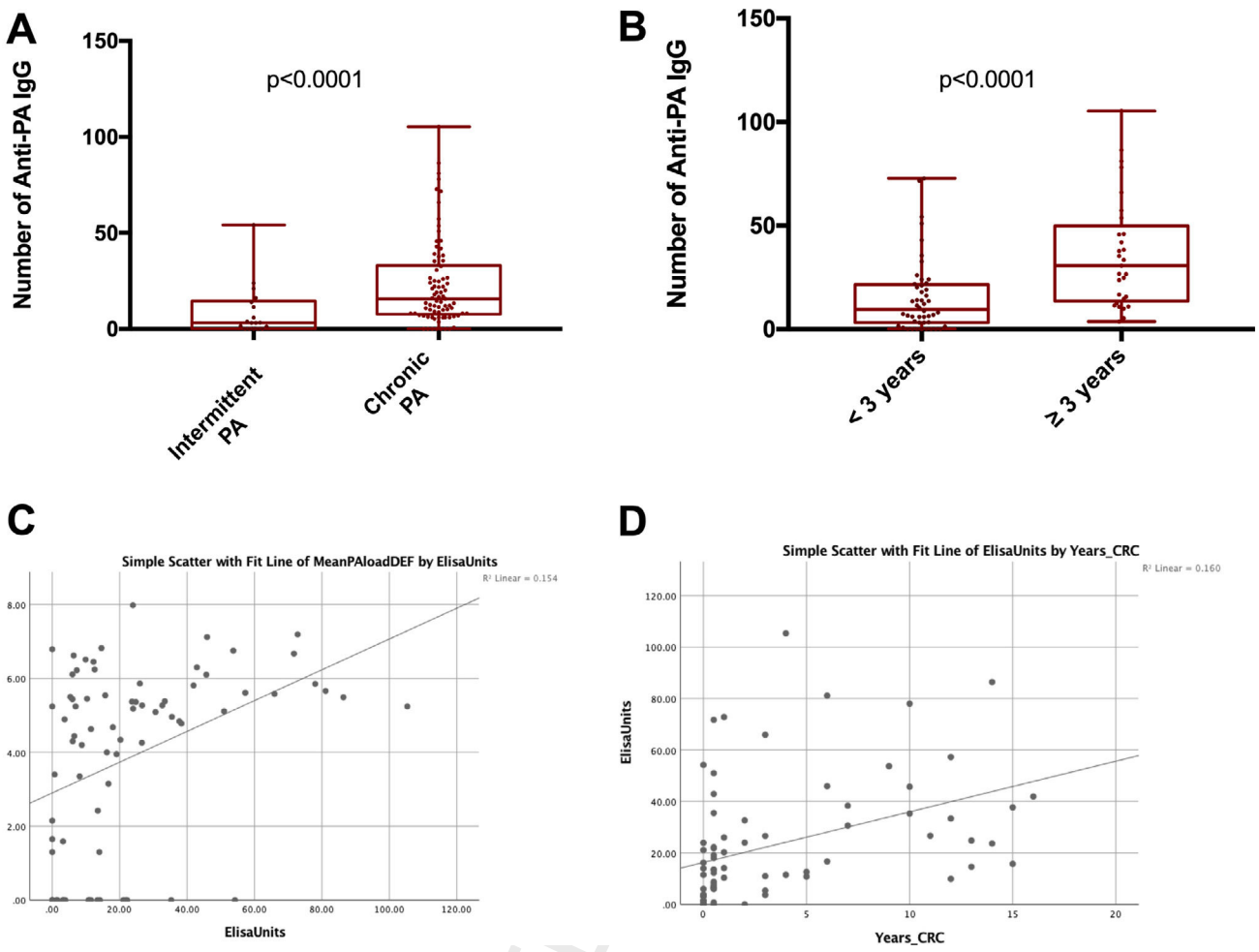


Fig. 4. AntiPA-IgG in serum samples from bronchiectasis (BE) patients. (A) AntiPA-IgG from BE patients with intermittent vs. chronic *P. aeruginosa* (PA) infection showed significant differences, being higher in the chronic group (median [IQR] 20.23 [9.89–38.33] vs. 3.25 [0.00–14.53], $p < 0.001$ respectively). (B) AntiPA-IgG levels were higher in patients with chronic infection of ≥3 years than in those with chronic infection of <3 years (median [IQR] 30.63 [13.58–49.82] vs. 9.55 [3.18–21.66], $p < 0.001$ respectively]. (C) Simple scatter plot (r:0.409; $p < 0.001$) charting the mean level of AntiPA-IgG and mean PA load. (D) Simple scatter plot (r:0.559; $p < 0.001$) charting the level of AntiPA-IgG up by years of chronic infection.

P. aeruginosa (Anti-PA IgG) were detected in chronic than in intermittently infected BE patients (Fig. 4A). Indeed, PA antibody levels increased in a time-dependent manner, being significantly higher in patients with ≥3 years since the diagnosis of chronic infection than in those with <3 years (Fig. 4B). We found a positive correlation between the mean level of systemic Anti-PAIgG and the mean PA load per patient (r:0.409; $p < 0.001$) (Fig. 4C). AntiPA-IgG positively correlated with years of chronic PA infection (r:0.559; $p < 0.001$) (Fig. 4D). Additionally, the systemic levels antiPA-IgG displayed high sensitivity for diagnosing chronic PA infection (Fig. 3S).

Impact of Chronic PA on Clinical Outcomes

After 1-year follow-up, chronic PA infection was associated with a decrease in hospitalized exacerbations (Fig. 5A). Similarly, higher levels of AntiPA-IgG were significantly associated with lower number of hospitalized exacerbations (Fig. 5B, E), No differences in predicted FEV_1% were found between chronically and intermittently colonized groups, either at inclusion (Table 1) or after 1-year follow-up (58.33 ± 27.09 vs. 62.87 ± 19.43, $p = 0.481$ respectively). After 1-year follow-up patients with intermittent PA infection improved in vitality, respiratory symptoms and emotional function domains of QoL. In contrast, chronically infected patients deteriorated in the emotional function and social domains of QoL (Fig. 5F).

Further, multivariable analyses showed that PA load and the mucoid phenotype were factors independently associated to chronic infection, but not the number of previous exacerbations (Table 2).

Despite two main clusters were found according to infection status (Fig. 5C, chronic or intermittent) or to PA phenotype (Fig. 5D, non-mucoid or mucoid), highlighted in the circle are the 25 patients with chronic/non-mucoid PA (Fig. 5D) who, interestingly, presented lower PA load (LogCFU/mL) (4.20 [0.00–5.67] vs 5.38 [4.84–6.10], $p = 0.015$ respectively), fewer years of chronic infection (1.0 [0.5–4.5] vs. 5.0 [0.5–12.0], $p = 0.014$ respectively),

are significantly more abundant in one group. Negative (red bars) LDA scores represent the most abundant bacterial groups in mucoid samples, while positive (green bars) represent those in non-mucoid samples. The bar size represents the effect size of the taxa within each group. Each sample is represented by a dot: blue dots represent the non-mucoid phenotype found in sputum, and red dots represent the mucoid phenotype. Samples with similar compositions appear in clusters; the two groups appear to present compositional differences. (D) Comparisons between Mucoid pathobiome No/Yes in terms of PALoad (CFU/mL), AntiPA-IgG and Years of chronic infection. The mucoid pathobiome was significantly associated with higher PA Load, higher AntiPA-IgG and more years of chronic PA infection.

and a trend towards lower levels of antiPA-IgG (13.80 [8.28–25.95] vs 26.00 [11.48–42.90], $p = 0.184$ respectively) compared to those with chronic/mucoid PA. Overall, these findings elucidate important factors involved in the transition from intermittent to chronic infection status and displayed in Fig. 5G.

Discussion

The main findings of our study are that patients with bronchiectasis and intermittent or chronic *P. aeruginosa* colonization present significant differences in clinical outcomes after 1-year follow-up.

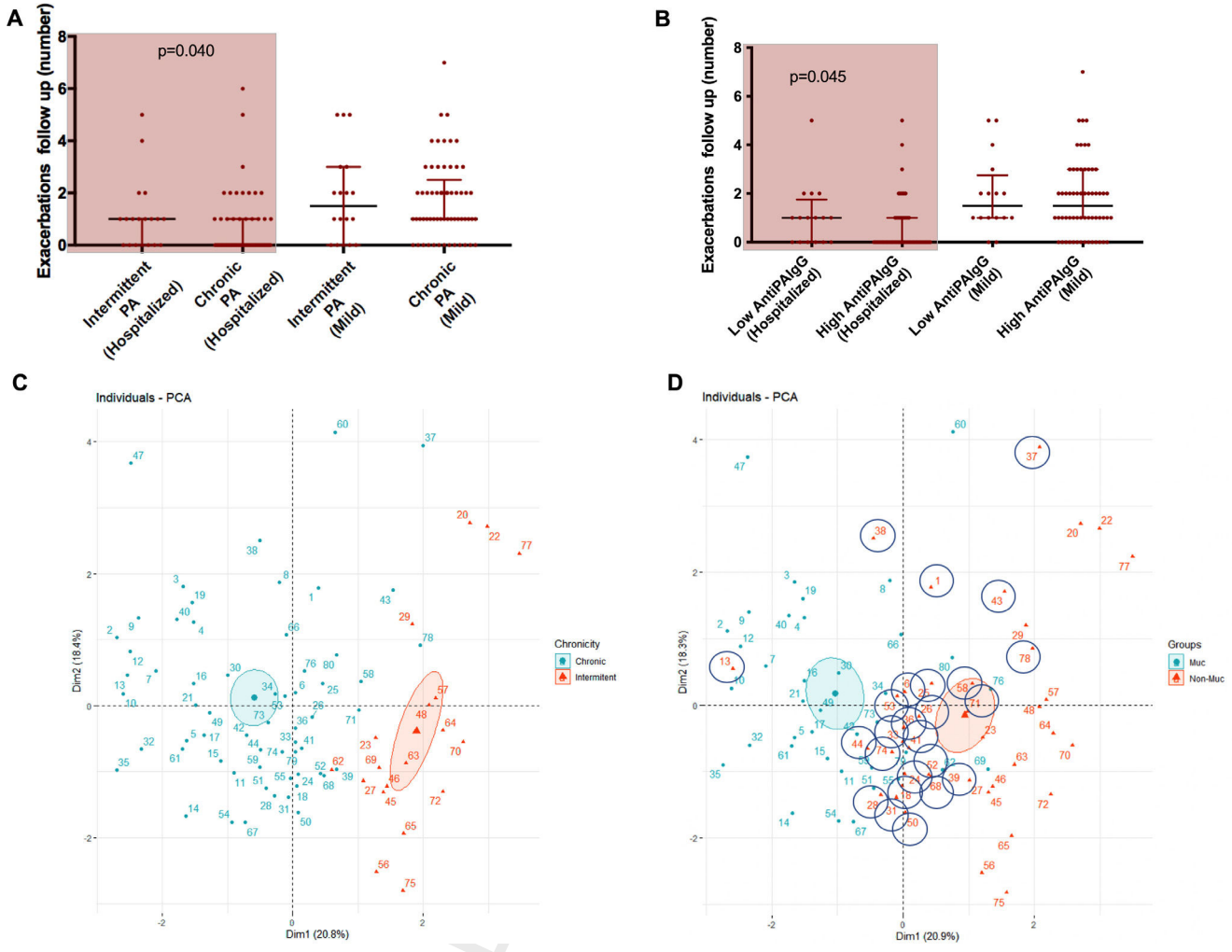


Fig. 5. Clinical and biological outcomes of BE patients with chronic vs intermittent *P.aeruginosa* colonization. (A) Number of hospitalized exacerbations during follow-up in BE patients with intermittent vs. chronic PA respiratory infections. Chronic PA infection ($n = 61$) was associated with a lower number of hospitalized exacerbations compared to those with intermittent colonization ($n = 19$) (0.00 [0.00–1.00] vs 1.00 [0.00–1.00], $p = 0.040$, respectively). The number of mild exacerbations did not differ between chronic vs. intermittent PA groups (1.00 [1.00–2.50] vs. 1.50 [0.00–3.00], $p = 0.947$). (B) Number of hospitalized exacerbations during follow-up in BE patients with low (<5) vs. high (≥5) systemic AntiPA-IgG. Patients with high AntiPA-IgG ($n = 61$) presented fewer hospitalized exacerbations than those with low AntiPA-IgG ($n = 16$) (0.00 [0.00–1.00] vs 1.00 [0.00–1.75], $p = 0.045$). The number of mild exacerbations did not differ between patients with high vs. low AntiPA-IgG (1.50 [1.00–3.00] vs. 1.50 [1.00–2.50], $p = 0.685$ respectively). C and D) Principal Component Analysis (PCA) analysis plot to assess the dissimilarity in relevant variables (*years of chronic infection, number of exacerbations (previous and follow-up, mild and hospitalized), FEV_1, mean PA load, Anti-PA-IgG*) between chronic (green) and intermittent (red) patients (C) or mucoid (green) and non-mucoid (red) *P. aeruginosa* isolation in sputa (D) or between females and males (Fig. 3S). NB: Circled Patients with chronic PA status but with non-mucoid PA in sputa are circle. Shaded ellipses indicate the confidence intervals. (E) Principal Component Analysis (PCA) plot showing the multivariate variation among 80 patients in terms of clinical variables. The plot shows the interplay between all clinical variables included in this multivariate analysis. Vectors indicate the direction and contribution of each clinical variable to the overall distribution. Coloured symbols correspond to the five most contributing variables identified in this study. The two principal axes explained the variance, in Dim1 (38.1%) and Dim2 (28.6%), respectively. Positively correlated variables are grouped together when the angle between them is less than 90°. In contrast, variables are non-correlated or negatively correlated when positioned on opposing quadrants of the plot and the angle between them is above 90° (contiguous or opposing quadrants). (F) Quality of life domains with significant changes after the 12-month follow-up period by colonization status, where the mean is marked with a red diamond. Sixty-nine patients were included in the QoL assessment: 49 (71%) with chronic PA colonization vs. 20 (29%) intermittent. When assessing the minimal important difference, we observed improvements in vitality (mean differences between visits 1 and 4 were 19.70, greater than the threshold of 10 points), emotional function (mean differences = 12.12, threshold of 7 points), and respiratory symptoms (mean differences = 9.09, threshold 8 points) in patients with intermittent colonization, whereas patients with chronic colonization recorded a loss of QoL in emotional (mean differences = −7.84, threshold 7 points) and social function (mean differences = −11.76, threshold 9 points). Additionally, the chronic vs. intermittent emotional function domain showed significant impairment at the 1-year follow-up (median 0.00 [0.00–33.33] vs. 0.00 [−16.67–0.00], $p = 0.036$). (G) Intermittent versus chronic *Pseudomonas aeruginosa (PA)* colonization. This figure illustrates the biological changes during the transition from intermittent to chronic PA colonization status. Intermittent PA colonization (left) is characterized by the non-mucoid phenotype growing in planktonic form indicated by the PA flagellum, microbiota biodiversity in lungs, low systemic levels of AntiPA-IgG and normal viscoelastic propierties of mucus. As time with intermittent colonization progresses without successful PA eradication, the colonization becames chronic (early stage) in which non-mucoid PA load increases although few mucoid PA colonies may be present and a trend to higher AntiPA-IgG is observed than in intermittent colonization. Finally, chronic PA colonization (right) is characterized by increased PA load and increased presence of the mucoid phenotype growing in biofilms (without flagellum). When chronic PA has been established the low biodiversity lung microbiota, with a predominance of *Proteobacteria*, and the increased mucus viscoelasticity complicates mucus clearance. In this context, systemic AntiPA-IgG levels are higher than in intermittent colonization.

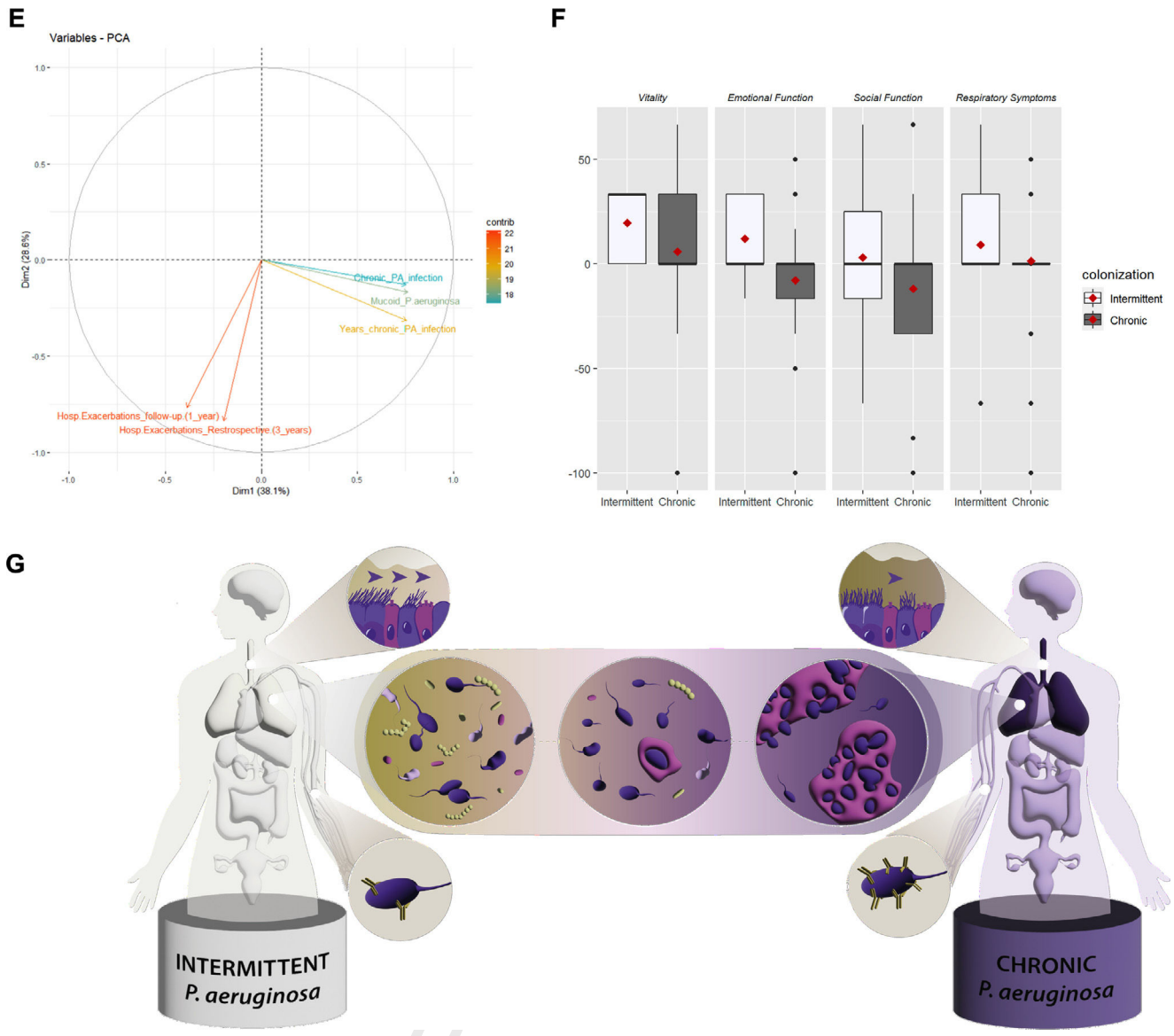


Fig. 5. (*Continued*)

Table 2
Univariate and Multivariable Logistic Regressison Analyses for Factors Independently Associated With Chronic Infection.

	Univariate			Multivariable[a,b]		
Variable	OR	95% CI	*p*-Value	OR	95% CI	*p*-Value
Male	0.72	0.26–2.03	0.536	0.70	0.18–2.75	0.613
Age (+1 year)[c]	0.99	0.94–1.04	0.650	1.01	0.95–1.08	0.725
Previous hospitalized exacerbation (+1)[c,d]	0.96	0.79–1.16	0.655	0.92	0.70–1.20	0.525
Mean PA load (+1)[c,e]	1.54	1.23–1.94	<0.001	1.39	1.08–1.79	0.012
Mucoid phenotype[f]	12.24	2.59–57.75	0.002	6.44	1.19–34.82	0.031

Abbreviations: CI: confidence interval; OR: odds ratio; PA: *Pseudomonas aeruginosa*. Data are shown as estimated ORs (95% CIs) of the explanatory variables in the chronic infection group. The OR represents the odds that the presence of chronic infection will occur given exposure of the explanatory variable, compared to the odds of the outcome occurring in the absence of that exposure. The *p*-values are based on the null hypothesis that all ORs relating to an explanatory variable equal unity (no effect).

[a] Hosmer–Lemeshow goodness-of-fit test, $p = 0.176$.
[b] Area under the ROC curve, AUC = 0.82 (95% CI 0.71–0.94).
[c] +1 year indicates a 1-unit increase.
[d] Exacerbations that required hospitalization were recorded during the three previous years.
[e] *P. aeruginosa* load was measured at each follow-up culture and reported as mean Pa load.
[f] Mucoid phenotype was defined if the mucoid *P. aeruginosa* was isolated from at least one of the follow-up cultures.

In particular, chronically infected patients had reduced quality of life but less hospitalized exacerbations. Additionally, the mucoid *P. aeruginosa* was a factor independently associated to chronic infection. Finally, patients chronically infected with the mucoid phenotype presented a significant loss in respiratory microbiome biodiversity.

Despite compared with other pathogens, *P. aeruginosa* infection is associated with more severe bronchiectasis and increased risks of mortality and hospital admissions,[1,21–23] recent reports have associated *Pseudomonas* or *Haemophilus* with clinical stability.[24,25] In addition, others found higher prevalence of *Pseudomonas* spp. in BE patients with low exacerbations and lower microbial interactions compared to those with high exacerbations and higher microbial interactions.[26] What our data adds to these previous reports is that PA chronic versus intermittent infection stage can explain the different exacerbation patterns displayed by BE patients with PA infection.

Our findings suggest the decreased number of hospitalized exacerbations found in chronically infected patients, compared to those with intermittent infection, could be associated with the high circulating AntiPA-IgGs levels found in the chronic ones. This could be a plausible explanation of our findings given that decreased IgG have been associated to increased exacerbations.[27] The association between new strains and increased risk of exacerbations has been reported in COPD patients[28–30] but deserves further research in BE patients.[14,31–33] Noteworthy, the chronic compared to intermittent PA infection was associated to a notable decline in the emotional, social, and vitality domains of quality of life, after one-year follow-up. The comparison of quality of life between these two groups has not been previously reported although chronic infection with PA has been associated to worse quality of life compared to non-PA infections.[3]

The mucoid PA phenotype was found to be a factor independently associated to chronic infection. In this context, ESCMID's methods for biofilm-associated infections enhance the sensitivity of PA identification useful not only for determining with higher accuracy those patients meeting the chronic infection definition (1.33 increase in newly diagnosed compared to SOCT) but also increasing PA detection during exacerbations (1.38 increase in PA detection compared to SOCT). In addition, at this chronic disease stage we demonstrated the presence of PA biofilms shown in Fig. 2 (microscopy images) and Table 1 (as means of mucoid phenotype and alginate presence) as well as its association to the mucoid phenotype (Fig. 4S). PA biofilms have recently been shown in paediatric patients with BE suggesting antibiofilm therapies could exhibit important benefits for disease progression whilst hindering the emergence of ciprofloxacin resistant PA which are highly prevalent in BE adult patients.[13,34]

P. aeruginosa pathophysiology in BE patients resemble that reported in CF since similar data to that reported in CF as regards increased AntiPA-IgG, as chronic colonization progresses, and PA biofilms were found in our population of non-CF bronchiectasis.[10,14,35] Previously, in vitro studies found biofilm related similarities comparing *P. aeruginosa* isolates from BE and CF confirming the similar pathophysiologic patterns displayed by *P. aeruginosa* between these two Chronic respiratory diseases.[36,37]

Our findings clustered apart two type of chronically colonized patients: those with the non-mucoid PA and those with the mucoid PA. The last ones indicative of a more advanced disease stage in which we found higher PA burden, increased AntiPA-IgG levels, more years of chronic colonization and significant loss in respiratory microbiota biodiversity (Fig. 5C and D). In accordance, a respiratory pathobiome dominated by *Proteobacteria* was characteristic of chronically colonized patients with the mucoid PA. In contrast, a microbiota with genera such as *Prevotella*, *Streptococcus* and *Veillonella*, representative of a healthy respiratory microbiota, was still present in the intermittently colonized group with the non-mucoid PA.[38] Differentiating between intermittent and chronic *P. aeruginosa* colonization is crucial, as successful eradication during the intermittent stage may help prevent progression to chronic infection. In this regard, our study suggests that the presence of the mucoid *P. aeruginosa* phenotype and systemic levels of anti-*P. aeruginosa* IgG may serve as valuable markers for diagnosing chronic colonization.

A major strength of our work is that we followed a cohort of BE patients colonized by *P. aeruginosa* with no significant clinical differences at baseline between intermittent vs. chronic PA infection as reported in Table 1, a feature that is crucial for avoiding other confounding factors. In addition, the long-term follow-up and sequential sampling (235 sputum and 262 serum samples) added an important value for establishing associations. The sample size (80 patients with positive isolation of *P. aeruginosa*) represents 12% of the screened population, a proportion which is in accordance with literature. The single centre nature of our study can be considered a limitation however it also represented the advantage of avoiding the bias of different heterogeneous cohorts, methods and ethiologies found in different countries.

In summary, in BE similar to CF patients the establishment of PA through biofilms during chronic infection deserves attention, since it causes not only a deterioration in quality of life and a loss in respiratory biodiversity but higher long-term mortality reported by others.[24] From this perspective, multiplex sydromic panels could also play an important role for the early PA detection, although they do not currently distinguish PA mucoid than non-mucoid phenotype. For the identification of the *P. aeruginosa* phenotype ESCMID methods for biofilms showed higher accuracy than SOCT.

Overall, our study reveals that in patients with bronchiectasis, the stage of *P. aeruginosa* infection, whether intermittent or chronic, correlates with variations in exacerbation patterns, quality of life, and respiratory microbiome composition. Our findings emphasize the significance of the PA mucoid phenotype, indicative of a chronic infection stage and associated with a distinct immunological response characterized by higher systemic levels of AntiPA-IgG antibodies. The potential role of antibodies against *P. aeruginosa* in mitigating the occurrence of severe exacerbations in patients with bronchiectasis and PA infection deserves further research.

Online Data Supplement

Any methods, additional references, source data, extended data, supplementary information, acknowledgements, peer review information; details of author contributions and competing interests; and statements and code availability are available in the online content of this article.

Contributorship

LFB and AT designed the protocol and secured adequate funding. LFB, RLA, RP, VA and LB participated in the data analysis and materials section. All authors participated either in the clinical management of patients or in data and sample collection and experimental work. LL and NH conducted the anti-PA IgG measurements in serum. VA and HSF performed the rheological measurements. The bioinformatic microbiome analysis was carried out by RLA and RP. LFB wrote the manuscript. All authors participated in the critical review of the submitted manuscript.

Ethics Approval

The study was approved by the Internal Ethical Review Board of the Hospital Clinic of Barcelona (registry number HCB/0236).

Data Sharing

The datasets analyzed for the current publication are available from the corresponding authors upon reasonable request, subject to material transfer agreements (MTAs). All data generated or analyzed during this study related to microbiome and PCA for clinical data were included in Github repository (https://github.com).

AI Use Statement

AI was not used in the creation of the manuscript's content.

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Conflict of Interest

A. Torres has received grants from MedImmune, Cubist, Bayer, Theravance and Polyphor, and personal fees as an advisory board member from Bayer, Roche, The Medicines CO and Curetis. He has received bureau fees for keynote speaker presentations from GSK, Pfizer, Astra Zeneca and Biotest Advisory Board, but these were not associated with the study described in this paper. The other authors have nothing to declare.

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Appendix A. Supplementary Data

Supplementary data associated with this article can be found, in the online version, at doi:10.1016/j.arbres.2025.03.003.

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