



# Isolation of and risk factors for airway infection with *Pseudomonas aeruginosa* in patients with non-cystic fibrosis bronchiectasis

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## ABSTRACT

**Objective:** To identify microorganisms in sputum samples of patients with stable non-cystic fibrosis bronchiectasis and to determine risk factors related to the isolation of *Pseudomonas aeruginosa* (PA) in those patients. **Methods:** Consecutive patients were recruited from a tertiary hospital outpatient clinic in the city of Fortaleza, Brazil. The patients were submitted to spirometry, six-minute walk test, HRCT, and sputum collection. Data on serum fibrinogen levels, disease severity, sputum color, and history of azithromycin treatment were collected. **Results:** The study included 112 patients, and females predominated (68%). The mean age was  $51.6 \pm 17.4$  years. Most patients presented with mild-to-moderate disease (83%). The mean six-minute walk distance was  $468.8 \pm 87.9$  m. Mean FEV<sub>1</sub> and FVC, in % of predicted values, were  $60.4 \pm 21.8\%$  and  $69.9 \pm 18.5\%$ , respectively. The mean serum fibrinogen level was  $396.1 \pm 76.3$  mg/dL. PA was isolated in 47 patients, other potentially pathogenic microorganisms (PPMs) were isolated in 31 patients, and non-PPMs were isolated in 34 patients. Purulent sputum was identified in 77 patients (68%). The patients with PA, when compared with those without it, presented with more severe disease, higher serum fibrinogen levels, and lower FVC%. In addition, purulent sputum and long-term azithromycin treatment were more common in those with PA. The multivariate regression analysis showed that the independent factors associated with PA were serum fibrinogen level  $> 400$  mg/dL (OR = 3.0; 95% CI: 1.1-7.7) and purulent sputum (OR = 4.3; 95% CI: 1.6-11.3). **Conclusions:** In our sample, the prevalence of PA in sputum was 42%. Sputum color and inflammatory markers were able to predict the isolation of PA, emphasizing the importance of routine sputum monitoring.

**Keywords:** Bronchiectasis; *Pseudomonas aeruginosa*; Sputum/microbiology.

## INTRODUCTION

Bronchiectasis is a growing health problem worldwide. The disease prevalence has increased by more than 40% in Europe and the USA in the past 10 years.<sup>(1)</sup> Non-cystic fibrosis bronchiectasis is characterized by non-reversible bronchial dilatation, usually accompanied by cough, sputum production, and recurrent respiratory infections.<sup>(2)</sup>

Chronic bacterial infections are often present in patients with bronchiectasis, contributing to the maintenance of the vicious circle of inflammation and progressive destruction of airways. Systemic inflammation is crucial for disease progression, and that can be associated with more adverse events and worse outcomes. There are various inflammatory markers that can be used for evaluation of disease progression, including interleukins, TNF- $\alpha$ , C-reactive protein (CRP), and fibrinogen.<sup>(3)</sup> Inflammation is associated with airway bacterial infection and may be responsible for airway destruction and loss of lung function. *Haemophilus influenzae* and *Pseudomonas*

*aeruginosa* have been the most common potentially pathogenic bacteria in bronchiectasis.<sup>(3,4)</sup>

Murray et al.<sup>(5)</sup> developed a quick and easy qualitative method to identify sputum color in patients with stable bronchiectasis. The sputum color chart uses photographs of sputum from patients with bronchiectasis, providing accurate representation of three major color grades, and showed good interobserver reliability between the doctor and the patient. Bacterial infection causes a pronounced increase in inflammatory markers that might be reflected by sputum purulence. This characteristic can be explored by clinicians using a sputum color chart.

Effects of *P. aeruginosa* on airway destruction might be indirectly reflected by lung function impairment.<sup>(6)</sup> Guan et al.<sup>(7)</sup> reported that a group of 144 patients with bronchiectasis and isolates of or infection with *P. aeruginosa* had poorer spirometry results. A recent study involving 186 patients followed at a bronchiectasis tertiary referral center in Portugal reported that patients with

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chronic bacterial infection had worse lung function when compared with those without that type of infection.<sup>(8)</sup>

Most studies on this topic have addressed the diversity of isolates of potentially pathogenic microorganisms (PPMs).<sup>(9,10)</sup> In patients with bronchiectasis, the few available data are mainly based on *P. aeruginosa* infection.<sup>(11)</sup> There is insufficient knowledge on *P. aeruginosa* infection, its associations, and useful clinical methods to distinguish *P. aeruginosa* from other PPMs.

Because of the importance of identifying bronchiectasis patients who are potentially infected with *P. aeruginosa*, new severity scores have been developed, such as FACED—an acronym for FEV<sub>1</sub>, Age, Chronic colonization with *P. aeruginosa*, Extent (of CT findings), and Dyspnea.<sup>(12)</sup>

Based on the hypothesis that patients infected with *P. aeruginosa* are a distinct group of patients within the group of patients with non-cystic fibrosis bronchiectasis, the objective of the present study was to assess the prevalence of *P. aeruginosa* isolation/infection in outpatients with bronchiectasis. Moreover, we expected that positive results could be associated with factors such as inflammatory markers and sputum color, as well as with clinical, radiological, and lung function parameters.

## METHODS

This was a cross-sectional study involving a group of adult patients with non-cystic fibrosis bronchiectasis who were consecutively selected from an outpatient clinic of a tertiary hospital between March of 2018 and October of 2019. Bronchiectasis was diagnosed on the basis of chest HRCT performed within the previous 12 months. Eligible patients had to remain exacerbation free for four weeks. Exacerbation was defined as the presence or worsening of three or more of the following key symptoms for at least 48 h: cough; high sputum volume/consistency; purulent sputum; breathlessness; exercise intolerance; fatigue; malaise; and hemoptysis.<sup>(2)</sup> The study protocol was approved by local research ethics committee (Protocol no. 1.844.662). All participants gave written informed consent.

We collected data on demographics; history of childhood respiratory infections (pertussis, pneumonia, and measles); history of pulmonary tuberculosis; diagnosed asthma, COPD, connective tissue disorders, and immune deficiencies; smoking status; history of long-term use of azithromycin; and treatment at the time of the last evaluation in a clinically stable phase. The participants were assessed regarding perception of dyspnea (modified Medical Research Council dyspnea score), sputum purulence/color (sputum color chart),<sup>(5)</sup> severity of bronchiectasis (FACED score),<sup>(12)</sup> and lung function (spirometry). Clinical assessment was performed by the attending doctor. Serum fibrinogen levels were also measured and compiled.

Samples of spontaneous sputum were obtained from all of the patients in the morning of their clinical

visit. Gram-stained smears of the samples showing  $\geq 25$  leukocytes/field and  $\leq 10$  epithelial cells/field (magnification,  $\times 100$ ) were considered valid sputum samples and processed for qualitative culture for bacteria (including AFB) and fungi. All microbiological samples were plated on blood agar, chocolate agar, Wilkins-Chalgren agar, Löwenstein-Jensen medium, and Sabouraud agar. In addition, the samples were smeared for Ziehl-Neelsen staining. The cultures were evaluated for growth after 48 h. Negative bacterial cultures were discarded after 5 days, negative fungal cultures were discarded after four weeks, and Löwenstein-Jensen cultures were discarded after six weeks. Bacterial/fungal load ( $\times 10^5$  CFU/mL) was calculated when a PPM was isolated. On the basis of culture results, the patients were divided into PA group (*P. aeruginosa*), PPM group (other than *P. aeruginosa*), and non-PPM group. The sputum color chart was shown to the patients so that they could identify one of the three typical color grades: clear (mucoid), pale yellow/pale green (mucopurulent), and dark yellow/dark green (purulent).<sup>(5)</sup>

HRCT scans were assessed for the number of lobes involved (the lingula was considered a separate lobe) and the most common type of bronchial dilatation (cylindrical, varicose, or cystic).<sup>(13)</sup>

Spirometry was performed using an electronic spirometer (WinDX; (Creative BioMedics Inc., San Clemente, CA, USA) in accordance with the American Thoracic Society/European Respiratory Society guidelines,<sup>(14)</sup> and FEV<sub>1</sub> and FVC results were collected and analyzed.

Exercise capacity was evaluated using the six-minute walk test, which measures the distance that a participant can walk on a flat 30-m corridor in six minutes.<sup>(15)</sup> The patients, under direct supervision of one of the investigators, were asked to walk as fast as possible from one end of the corridor to the other, as many times as possible, within the established time. All patients performed two tests, with a minimum time interval of 30 min, and the best result was recorded.

The FACED score has been used as a tool to assess the severity of bronchiectasis.<sup>(12)</sup> As previously mentioned, the score incorporates five dichotomous variables, and the scores of each variable are summed up to provide the total score, which can range from 0 to 7 points. The total score classifies bronchiectasis into three levels of severity: mild (0-2 points), moderate (3-4 points), and severe (5-7 points). The FACED score has been validated for use in Brazil.<sup>(16)</sup>

## Sample size

A previous study showed that the prevalences of *P. aeruginosa* and other microorganisms in patients with bronchiectasis were 15% and 40%, respectively.<sup>(17)</sup> For the purpose of multivariate logistic regression analysis, the dependent variable was dichotomized as PA group or non-PA group (i.e., PPM + non-PPM groups), assuming that the rates of these groups were 15% and 40%,

respectively. Defining  $\alpha < 0.05$  and  $\beta < 0.20$ , at least 57 participants were required for one arm.

**Statistical analysis**

Categorical variables were described as absolute and relative frequencies, whereas continuous variables were described as mean and standard deviation, when appropriate.

One-way ANOVA was used in order to compare the means of the three groups individually, followed by a post hoc analysis with Bonferroni correction to clarify the differences between the pairs of groups (PA vs. PPM; PA vs. non-PPM; and PPM vs. non-PPM). For comparison of proportions, the chi-square test with post hoc analysis for pairwise comparisons was used with Bonferroni-adjusted p value.<sup>(18)</sup> As previously mentioned, the dependent variable was dichotomized as PA group or non-PA group. The independent factors selected for the multivariate analysis were those considered to be clinically relevant or potential confounders for the identification of PA isolates: sex (female); FVC ( $< 80\%$  of the predicted value); sputum color<sup>(5)</sup> (purulent); serum fibrinogen level ( $> 400$  mg/dL); and FACED total score<sup>(12)</sup> ( $\geq 5$ ). Multicollinearity was assessed using the variance inflation factor (VIF); a VIF  $< 2.5$  was regarded as an exclusion of any significant interaction.<sup>(19)</sup> The results were reported as OR and 95% CI. The significance level was set at  $p < 0.05$ . All statistical analyses were performed with the IBM SPSS Statistics software package, version 21.0 (IBM Corporation, Armonk, NY, USA).

**RESULTS**

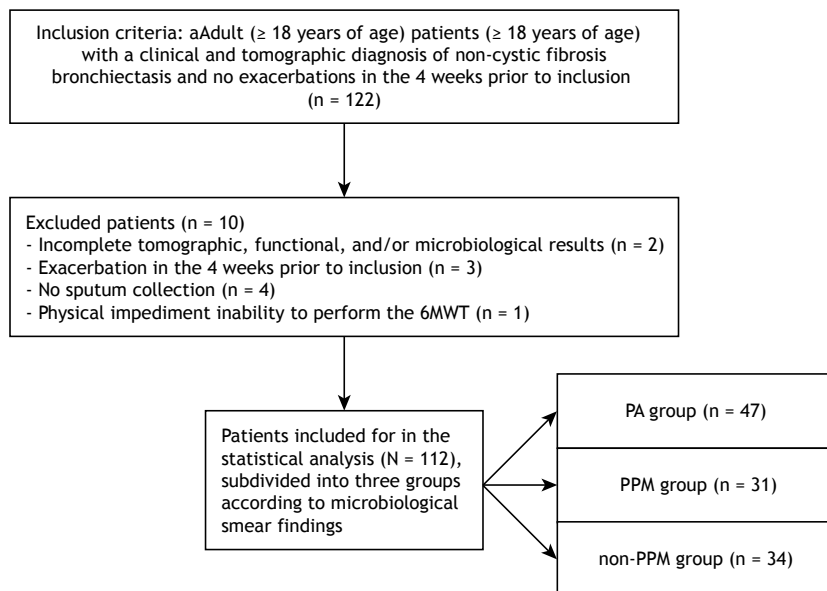
The flow chart of patient recruitment is shown in Figure 1. A total of 122 consecutive patients with non-cystic fibrosis bronchiectasis were initially included

in the study. Of those, 10 did not meet the inclusion criteria and were excluded. Therefore, the total sample comprised 112 patients.

Table 1 shows that 77 patients (68%) were female. The mean age was  $51.7 \pm 17.4$  years. According to the FACED score, 83% of the patients presented with mild-to-moderate disease. Mucopurulent/purulent sputum predominated ( $n = 77$ ; 68%). Of those 77 patients with mucopurulent/purulent sputum, 70 (62% of the total sample) were submitted to long-term azithromycin treatment (500 mg, three times/week).

The etiology of bronchiectasis was determined by means of the review of clinical medical records. An underlying etiology was identified in 65% of the patients. In 35% of the patients no cause was established (classified as idiopathic bronchiectasis). The remaining etiologies were described as post-tuberculosis bronchiectasis, in 30%; post-infection bronchiectasis, in 5%; Kartagener syndrome, in 8%; and other etiologies, in 22%.

The microorganisms identified in the sputum of the patients are detailed in Table 2, whereas Table 3 shows the comparison of the selected variables between the groups (PA, PPM, and non-PPM). Significant differences were found regarding the following variables: serum fibrinogen levels, which were higher in the PA group when compared with the PPM group ( $425.4 \pm 78.3$  mg/dL vs.  $380.5 \pm 72.2$  mg/dL;  $p = 0.04$ ) and the non-PPM group ( $425.4 \pm 78.3$  mg/dL vs.  $357.4 \pm 75.5$  mg/dL;  $p = 0.001$ ); FVC in % of predicted values, which was lower in the PA group when compared with the PPM group ( $64.3\% \pm 16.5\%$  vs.  $75.9\% \pm 14.7\%$ ;  $p = 0.02$ ); proportion of patients with purulent sputum, which was higher in the PA group when compared with the PPM ( $66.0\%$  vs.  $32.3\%$ ;  $p = 0.003$ ) and non-PPM groups ( $66.0\%$  vs.  $14.7\%$ ;  $p < 0.001$ ); severe bronchiectasis,



**Figure 1.** Flow chart of patient recruitment. 6MWT: six-minute walk test; PA: *Pseudomonas aeruginosa*; and PPM: potentially pathogenic microorganisms (other than *P. aeruginosa*).

**Table 1.** Characteristics of the patients.<sup>a</sup>

Variable	(N = 112)
Sex	
Female	77 (68)
Male	35 (31)
Age, years	51.7 ± 17.4
Smoking status	
Never smokers	81 (73)
Former smokers	31 (27)
BMI, kg/m <sup>2</sup>	22.5 ± 4.5
FACED score	3 [1-4]
Disease severity (FACED score)	
Mild	60 (53)
Moderate	34 (30)
Severe	18 (16)
Exacerbations in the last year	
0	40 (35)
1-2	54 (48)
> 3	18 (16)
Hospitalizations in the last year	
Yes	19 (17)
No	93 (83)
FEV <sub>1</sub> , % of predicted	60.4 ± 21.8
FVC, % of predicted	69.9 ± 18.5
FEV <sub>1</sub> /FVC	71.1 ± 15.0
6MWD, m	468.8 ± 87.9
Fibrinogen, mg/dL	396.1 ± 76.3
mMRC score	
0-1	45 (40)
≥ 2	67 (60)
Number of lobes involved	
< 2	8 (7)
≥ 2	104 (93)
Group	
PA	47 (42)
PPM	31 (27)
Non-PPM	34 (30)
Sputum color chart classification	
Mucoid	35 (31)
Mucopurulent	31 (27)
Purulent	46 (41)
Long-term azithromycin treatment	70 (62)

FACED: acronym for FEV<sub>1</sub>, Age, chronic Colonization by *Pseudomonas aeruginosa*, Extent (of CT findings), and Dyspnea; 6MWD: six-minute walk distance; mMRC: modified Medical Research Council (scale); PA: *P. aeruginosa*; and PPM: potentially pathogenic microorganisms (other than *Pseudomonas aeruginosa*). <sup>a</sup>Values expressed as n (%), mean ± SD, or median [IQR].

which was higher in the PA group when compared with the non-PPM group (29.8% vs. 2.9%;  $p = 0.002$ ); and long-term azithromycin treatment, which was more common in the PA group when compared with the non-PPM group (80.9% vs. 41.2%;  $p < 0.001$ ).

The multivariate logistic regression analysis was performed to determine the factors associated with the

*P. aeruginosa* isolates (Table 4). Independent variables were gender, FACED score, serum fibrinogen level, FVC%, and sputum color classification. All of the factors had a VIF < 2.0. The independent factors associated with the isolation of *P. aeruginosa* were fibrinogen > 400 mg/dL (OR = 3.00; 95% CI: 1.10-7.77) and purulent sputum (OR = 4.33; 95% CI: 1.60-11.38).

## DISCUSSION

The present cross-sectional study showed that, in our sample of patients with steady-state bronchiectasis, 47 (42%) harbored *P. aeruginosa* in the airways. The rate of *P. aeruginosa* isolates was significantly higher than was that of *H. influenzae*, corroborating the findings in a study by Guan et al.<sup>(7)</sup> Multivariate logistic regression analysis identified that high levels of serum fibrinogen and purulent sputum were associated with isolation of *P. aeruginosa*. We would like to emphasize that the use of the sputum color chart by Murray et al.<sup>(5)</sup> provided novel evidence about this rapid and practical way for clinicians to predict the presence of *P. aeruginosa* in the airways and distinguish it from other microbiological statuses. This useful tool indicates the severity of inflammation, airway destruction, and proteolytic enzyme activity/presence of neutrophilic airway disease, such as non-cystic fibrosis bronchiectasis, as well as COPD or asthma.<sup>(20-22)</sup>

The correlation of sputum color with positive cultures is not very clear, findings of positive and negative relationships having been described.<sup>(22-24)</sup> A recent meta-analysis<sup>(25)</sup> analyzing six studies on sputum staining and positive cultures in COPD patients showed that the isolation of bacteria in sputum is less likely to occur when sputum is classified as mucoid. More patients with purulent sputum presented with bacterial colonization than did patients with mucopurulent or mucoid sputum.

We found that the presence of a systemic inflammatory response (as evidenced by elevated circulating fibrinogen levels) was associated with the isolation of *P. aeruginosa*. Fibrinogen levels were higher in the PA group when compared with the PPM and non-PPM groups, which might explain the role of *P. aeruginosa* in systemic inflammation. Previous studies found that airways harboring *P. aeruginosa* showed significantly higher airway inflammation.<sup>(7,26-29)</sup>

Menéndez et al.<sup>(29)</sup> conducted a prospective observational study and found progressive increases in the levels of systemic proinflammatory cytokines and CRP in hospitalized patients with bronchiectasis from whom *P. aeruginosa* was isolated during acute and chronic phases of exacerbations. The level of systemic inflammation remained high after the acute phase. Jin et al.<sup>(30)</sup> found that systemic inflammatory markers, including CRP and fibrinogen, were significantly elevated in COPD patients with bronchiectasis. The use of other inflammatory markers is necessary to detect the severity of inflammation so that better treatment can be provided for patients with bronchiectasis. We

decided to measure serum fibrinogen levels, because serum fibrinogen is a biomarker for which routine measurements are available in clinical practice.

Ergan Arsava & Cöplü,<sup>(31)</sup> studied 50 patients with stable bronchiectasis and found that fibrinogen and

CRP levels were higher in those with airway colonization than in those without it. In a subgroup of patients colonized with *P. aeruginosa*, those levels were even higher than were those in their counterparts.

In our study, the presence of *P. aeruginosa* in the airways of patients was associated with reduced FVC. This could be explained by the effects of *P. aeruginosa* on inflammation and destruction of the airways. Studies have shown that when *P. aeruginosa* or *H. influenzae* dominates the microbiome of patients with bronchiectasis, their lung function is significantly reduced.<sup>(10,32)</sup>

The frequency of exacerbations and hospitalizations in the previous year in our sample of patients with *P. aeruginosa* was not statistically significant. This could be explained by the long-term use of azithromycin by these patients. A clinical trial conducted by Richardson et al.<sup>(32)</sup> demonstrated a significant reduction in the number of exacerbations in patients treated with erythromycin when compared with those treated with placebo. A meta-analysis of nine studies (530 patients) demonstrated that macrolide use reduced the number of patients with exacerbations and the

**Table 2.** Microorganisms isolated from sputum samples of patients with non-cystic fibrosis bronchiectasis (N = 112).

Isolate	n
<i>Pseudomonas aeruginosa</i>	47
Nonmucoid strain	28
Mucoid strain	19
<i>Haemophilus influenzae</i>	12
<i>Klebsiella pneumoniae</i>	9
<i>Moraxella catarrhalis</i>	1
<i>Streptococcus pneumoniae</i>	1
Methicillin-sensitive <i>Staphylococcus aureus</i>	1
Other PPM <sup>a</sup>	7
Non-PPM	34

PPM: potentially pathogenic microorganisms.  
<sup>a</sup>*Achromobacter xylosoxidans* (n = 2); *Aspergillus* sp. (n = 3); *Candida albicans* (n = 1); and *Mycobacterium tuberculosis* (n = 1).

**Table 3.** Comparisons between the groups formed according to the microbiological status of the patients.

Variable	Group			p
	PA (n = 47)	PPM (n = 31)	Non-PPM (n = 34)	
Age, years*	52.6 ± 19.0	53.8 ± 16.5	48.5 ± 16.0	0.430
Female*	32 (68.0)	19 (61.3)	26 (23.4)	0.400
BMI, kg/m <sup>2†</sup>	22.7 ± 5.0	21.9 ± 3.9	22.9 ± 4.5	0.650
Disease severity (FACED) <sup>†</sup>				
Mild/moderate	33 (70.0)	28 (90.3)	33 (97.1)	0.003
Severe	14 (29.8) <sup>‡</sup>	3 (9.7)	1 (2.9)	
Number of lobes involved				
< 2	3 (6.4)	3 (9.7)	2 (5.9)	0.810
≥ 2	44 (93.6)	28 (90.3)	32 (94.1)	
Serum fibrinogen, mg/dL*	425.4 ± 78.3 <sup>‡,§</sup>	380.5 ± 72.2	357.4 ± 75.5	0.001
FEV <sub>1</sub> , % of predicted*	55.1 ± 18.9	63.7 ± 21.8	62 ± 25.3	0.180
FVC, % of predicted*	64.3 ± 16.5 <sup>‡</sup>	75.9 ± 14.7	69.5 ± 22.7	0.020
6MWD, m*	457.5 ± 98.8	459.1 ± 85.8	493 ± 87.5	0.180
mMRC dyspnea scale score				
0-1	15 (31.9)	15 (48.4)	15 (44.1)	0.290
≥ 2	32 (68.1)	16 (51.6)	19 (55.9)	
Sputum color chart classification <sup>†</sup>				
Mucoid/mucopurulent	16 (34.0)	21 (67.7)	29 (85.3)	0.001
Purulent	31 (66.0) <sup>‡,§</sup>	10 (32.0)	5 (14.7)	
Exacerbations in the last year				
0-2	35 (74.5)	29 (93.5)	30 (88.2)	0.050
3-4	12 (25.5)	2 (6.5)	4 (11.8)	
Hospitalizations in the last year				
Yes	10 (21.3)	4 (12.2)	5 (14.7)	0.500
No	37 (78.7)	27 (87.1)	29 (85.3)	
Long-term azithromycin treatment	38 (80.9) <sup>‡</sup>	18 (58.1)	14 (41.2)	0.001

PA: *Pseudomonas aeruginosa*; PPM: potentially pathogenic microorganisms (other than *P. aeruginosa*); FACED: acronym for FEV<sub>1</sub>, Age, chronic Colonization by *P. aeruginosa*, Extent (of CT findings), and Dyspnea; 6MWD: six-minute walk distance; and mMRC: modified Medical Research Council. \*ANOVA with Bonferroni correction. <sup>†</sup>Chi-square test and post hoc analysis for pairwise comparisons. <sup>‡</sup>PA vs. non-PPM (p < 0.05). <sup>§</sup>PA vs. PPM (p < 0.05).

**Table 4.** Multivariate logistic regression analysis of factors associated with isolation of *Pseudomonas aeruginosa* in the sputum of patients with non-cystic fibrosis bronchiectasis.

Factor	OR	95% CI	p
Purulent sputum	4.33	1.60-11.38	0.003
Fibrinogen >400 mg/dL	3.00	1.10-7.77	0.020
Female	0.98	0.34-2.78	0.970
Severe disease	2.42	0.61-9.60	0.200
FVC < 80% of predicted	2.32	0.82-6.50	0.110

number of exacerbations per patient.<sup>(33)</sup> The small number of hospitalizations was probably related to the small number of exacerbations in our study.

More severe disease, measured by the FACED score, was associated with the isolation of *P. aeruginosa*. This is expected because this tool incorporates colonization by *P. aeruginosa* in its metrics, emphasizing the importance of chronic infection in the severity of bronchiectasis.<sup>(12)</sup>

The number of lobes involved on CT scans was not found to be associated with the isolation of *P. aeruginosa*. Therefore, we cannot rule out or confirm that the presence of *P. aeruginosa* is a factor related to greater radiological structural damage in these patients.

The limitations of the present study include the following: i) the sample size was small, but we were able to identify variables with biological plausibility; ii) patients were recruited at a referral facility, making

it difficult to extrapolate our results to other realities; iii) the follow-up period was short, and no molecular methods were used in order to understand the role that each microorganism plays in disease progression; and iv) the observational, cross-sectional design makes it difficult to establish temporal order and causal direction.

The present study is relevant because we might assume that a useful clinical method such as a sputum color chart<sup>(5)</sup> is able to predict airway infection with *P. aeruginosa*. Identifying sputum color in association with clinical manifestations of infection might be a useful strategy for clinicians to manage these patients while awaiting formal sputum microbiology results. In addition, the use of serum fibrinogen as a marker is a simple and reliable method to identify infected patients and should therefore be part of routine clinical practice. Larger multicenter longitudinal studies are needed to improve the characterization of other PPMs and their individual clinical impact.

## AUTHOR CONTRIBUTIONS

IL and AA: study design; data collection; literature search; and approval of the final version. MRF: study design; data collection; literature search; drafting of the manuscript; approval of the final version. FL: drafting of the manuscript; and approval of the final version. EDBP: study design; drafting of the manuscript; final revision; and approval of the final version.

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