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Extracellular DNA in sputum is associated with pulmonary function and hospitalization in patients with cystic fibrosis

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ABSTRACT

Background: Elevated extracellular DNA levels are found in the sputum of patients with cystic fibrosis (CF). However, studies investigating the association of extracellular DNA with CF severity are scarce.
Objective: To evaluate the association of extracellular DNA levels with pulmonary function, antibiotic use, and hospitalization in CF patients.
Methods: This cross-sectional study included CF patients aged ≥5 years who were clinically stable and produced spontaneously expectorated sputum. Extracellular DNA in sputum was quantified, and extracellular DNA networks were seen with immunofluorescence microscopy. Also, cell death profile was assessed. Data on pulmonary function, airway colonization, antibiotic use, and hospitalization in the previous year were collected. Patients were divided into two groups based on median DNA level.
Results: Thirty-three patients were included. Their mean age was 16.3 ± 6.2 years, mean forced expiratory volume in the first second (FEV_1) was 67.0 ± 26.7 (% of the predicted), and mean DNA level was 241.9 ± 147.2 μg/mL. There were significant correlations of DNA level with FEV_1 ($r = -0.60$; $p < 0.001$) and forced vital capacity ($r = -0.59$; $p < 0.001$). Moreover, patients with higher DNA level (>243.0 μg/mL) had lower FEV_1 (52.1 ± 27.8% *vs.* 81.1 ± 16.2%; $p = 0.001$) and required more hospitalizations (68.8% *vs.* 35.3%; $p = 0.05$). Additional findings were the presence of extracellular DNA networks and low rates of necrosis and apoptosis.
Conclusion: Elevated extracellular DNA levels in CF sputum are associated with reduced pulmonary function and increased hospitalizations.

1. Introduction

Lung disease is the leading cause of morbidity and mortality in cystic fibrosis (CF) [1]. Mucus collection caused by a dysfunction in the cystic fibrosis transmembrane conductance regulator (CFTR) protein favors airway colonization and infections, which contributes to frequent pulmonary exacerbations requiring antibiotics and often hospitalization [2,3]. Exacerbations have a strong impact on patient survival [4,5] and are known as an outcome measure [6] because of their contribution to impaired pulmonary function [5,7,8], poor exercise performance [9], and decreased quality of life [10].

In CF patients, airways present with chronic neutrophilic inflammation, which is considered a major factor for early development of lung disease [11]. Elevated extracellular DNA levels are found in the sputum and bronchoalveolar lavage samples from CF patients [12–14], deriving from necrotic and apoptotic leukocytes and especially from release of extracellular DNA networks, or neutrophil extracellular traps (NETs) [15–17]. DNA affects the rheological properties of mucus by changing fluid viscosity and elasticity, which contributes to airway obstruction [18]. DNA level in sputum is known to be negatively associated with pulmonary function and disease severity in CF patients [14,19].

The relationship between DNA and lung disease has already been investigated, but knowledge regarding NET involvement in CF is recent. Although NETs are released by neutrophils as a defense mechanism, NET excess is believed to lead to airway obstruction, bacterial colonization, and lung tissue damage [20]. However, studies investigating the

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association of extracellular DNA and NETs with CF severity are scarce. Thus, this study aimed to evaluate the association of extracellular DNA levels in sputum with pulmonary function, antibiotic use, and hospitalization due to pulmonary exacerbations in CF patients.

2. Materials and methods

A cross-sectional study was conducted to evaluate CF patients treated at 2 specialized Cystic Fibrosis centers from August 2018 to September 2019. Patients were included if they were aged ≥5 years, had reproducible spirometry, and were able to produce spontaneously expectorated sputum. Patients were excluded if they had an inadequate sputum sample, were using antibiotics, or presented with signs of pulmonary exacerbation in the past 15 days, including fever, increased cough, and increased sputum production [6]. A convenience sample was selected.

Sample size was determined based on data from a previous study that assessed the correlation between DNA and lung function [14]. An expected correlation of 0.6 between the variables, a significance level of 0.05, and a power of 95% were used to estimate that 30 participants were required.

The present study was approved by the Research Ethics Committees of both centers with the following protocol numbers 91277118.5.0000.5336 and 91277118.5.3002.5327. All legal guardians and subjects over the age of 18 years signed the informed consent form. Moreover, children and adolescents under the age of 18 years signed the assent form.

2.1. Study design

Patients with a history of expectorated sputum were identified from medical records or follow-up forms. For patients using dornase alfa (Pulmozyme®, Roche), waiting at least 12 h between nebulization and sputum collection was recommended because of the drug's action on DNA.

Sputum was produced spontaneously, i.e., without previous induction with hypertonic solution. The participants were instructed to clean their mouth beforehand to remove superficial bacterial flora and avoid sample contamination. Then they were asked to expectorate in a sterile container. If necessary, they were instructed to perform some respiratory cycles and the forced expiratory technique (huffing) followed by coughing in order to mobilize lower airway secretion. The samples were kept in a refrigerated environment for up to 2 h until processing and analysis.

Anthropometric and pulmonary function variables were recorded, and additional data were collected from medical records regarding genetic mutation, pancreatic function, airway colonization, number of exacerbations, days of antibiotic use, and hospitalization.

2.2. Procedures

2.2.1. Anthropometric data

Weight and height measurements were performed during the outpatient visit. Weight was measured in the orthostatic position using a digital scale, with a precision of 100 g, and height was obtained with a stadiometer (precision of 1 mm). Then, the body mass index (BMI) was calculated (weight/height^2).

2.2.2. Pulmonary function

Pulmonary function was assessed with spirometry according to American Thoracic Society/European Respiratory Society (ATS/ERS) criteria [21], which is routine practice in outpatient visits. The variables of interest were forced expiratory volume in the first second (FEV_1), forced vital capacity (FVC), FEV_1/FVC ratio, and forced expiratory flow between 25 and 75% of FVC ($FEF_{25–75\%}$). Data were normalized using the Global Lung Function Initiative (GLI-2012) reference equation [22].

2.2.3. Chronic colonization with Pseudomonas aeruginosa and exacerbations

Chronic colonization with *Pseudomonas aeruginosa* (PA) was defined as positive cultures in more than 50% of samples in the 12 months prior to the analysis [23]. Exacerbations were defined as the clinical need for either oral or intravenous (IV) antibiotics due to the presence of at least two of the following criteria: increased cough and expectorated sputum, change in secretion, abnormal radiological findings, dyspnea, malaise, and fatigue, weight loss, and decreased pulmonary function by more than 10% [6]. The number of days using oral and IV antibiotics and hospitalizations due to pulmonary exacerbations in the previous 12 months were quantified.

2.2.4. Sputum processing

The sputum sample (150–250 mg) was macroscopically separated from saliva and homogenized in dithiothreitol (DTT) and Dulbecco's phosphate-buffered saline (DPBS). Then it was passed through a nylon filter and centrifuged at 2000 rpm for 5 min. Histological slides were prepared in a cytocentrifuge and stained using the Fast Panoptic method (Laborclin), and differential leukocyte count was expressed as percentage of cell type. The resulting supernatant was stored at −20 °C for subsequent quantification of extracellular DNA.

2.2.5. DNA quantification and immunofluorescence

Extracellular DNA was quantified using a dsDNA HS assay kit (Invitrogen) and a Qubit 2.0 fluorometer (Invitrogen), according to the manufacturer's instructions. For visualization of DNA networks with immunofluorescence, sputum cells were left to adhere to an eight-well chamber (Nunc Lab-Tek, Thermo Fisher) previously coated with poly-L-lysine, as proposed by Manzenreiter et al. [24]. Then they were fixed with 4% paraformaldehyde, blocked with bovine serum albumin (1% BSA), and washed with phosphate-buffered saline (PBS). For DNA labeling, cells were stained with Hoechst 33,342 (1:2000, Molecular Probes). Images were obtained using fluorescence microscopy (BMX 43 microscope equipped with Olympus DP73 digital camera).

2.2.6. Cell death detection

Granulocytes in sputum were differentiated regarding apoptosis and necrosis with Annexin V-FITC and propidium iodide (PI) (BD Bioscience), respectively, and were analyzed with flow cytometry (FACS Canto II, BD Bioscience), according to the manufacturer's instructions.

2.2.7. Statistical analysis

The Kolmogorov–Smirnov test was used to assess normality of data. Data were expressed as mean and standard deviation or median and interquartile range, according to their distribution. Categorical variables were expressed as absolute and relative frequencies. Pearson's or Spearman's correlation coefficients were used to assess correlations between extracellular DNA in sputum and the variables of interest. For comparison purposes, patients were divided into two groups based on median DNA level in sputum (243.0 μg/mL), and Student's t-test for independent samples and Pearson's chi-square test were used. For multiple comparisons, a one-way ANOVA followed by the Bonferroni post-hoc test was used. All analyses were performed in SPSS 18.0 and Prism 5.0 (GraphPad Software), with a significance level set at 5% ($p \leq 0.05$).

3. Results

Forty-two patients were invited to participate in the study, and nine of them were excluded because their sputum sample was inadequate for analysis. Thus, the final sample consisted of 33 patients. Their mean age was 16.3 ± 6.2 years, and 51.5% were male. Table 1 shows the main characteristics of the sample. Extracellular DNA levels in CF sputum ranged from 44.22 μg/mL to 660.82 μg/mL, with a mean level of 241.9 ± 147.2 μg/mL. In differential cytological assessment, an important

Table 1
Characteristics of the study sample.

Variables	n = 33
Demographics	
Age (years)	16.3 ± 6.2
Male, n (%)	17 (51.5)
Anthropometric	
Weight (kg)	47.7 ± 14.6
Height (cm)	154.7 ± 18.3
BMI (kg/m^2)	19.3 ± 2.6
Clinical	
Age at diagnosis (years)	0.6 (0.2–5.3)*
Pancreatic insufficiency, n (%)	30 (90.9)
Chronic *Pseudomonas aeruginosa*, n (%)	9 (27.3)
Use of Dornase alfa, n (%)	31 (93.9)
Extracellular DNA in sputum (μg/mL)	241.9 ± 147.2
Genotyping	
F508del homozygous, n (%)	9 (27.3)
F508del heterozygous, n (%)	16 (48.5)
Other mutations, n (%)	8 (24.2)
Lung function	
FEV_1 (% predicted)	67.0 ± 26.7
FVC (% predicted)	79.2 ± 23.6
FEV_1/FVC (absolute)	0.72 ± 0.13
$FEF_{25–75\%}$ (% predicted)	45.7 ± 31.7

BMI: body mass index; FEV_1: forced expiratory volume in 1 s; FVC: forced vital capacity; $FEF_{25–75\%}$: forced expiratory flow between 25 and 75% of vital capacity. Values expressed as absolute frequency (relative) or mean ± standard deviation, except for age at diagnosis expressed as *median (interquartile range).

predominance of neutrophils was found (Fig. 1A and B). Additionally, immunofluorescence microscopy revealed the presence of extracellular DNA networks (Fig. 1C and D). As shown in Fig. 1E and F, flow cytometry demonstrated that cell viability remained high (91.3% and 73.9%, respectively), considering the low rates of necrosis (5.9% and 6.1%), apoptosis (1.5% and 10.8%), and late apoptosis/necrosis (1.2% and 9.2%).

Regarding pulmonary function variables, significant moderate negative correlations were found between extracellular DNA levels and percent FEV_1 ($r = -0.60$; $p < 0.001$), FVC ($r = -0.59$; $p < 0.001$), and $FEF_{25–75\%}$ ($r = -0.47$; $p = 0.006$). However, no significant associations were found with age, BMI, number of days using antibiotics, and hospitalization (Fig. 2).

Patients were divided into two groups based on median DNA level in the sample. Those with higher DNA concentration in sputum (>243.0 μg/mL) had lower FEV_1 values (Fig. 3A) compared to those with lower DNA concentration (52.1 ± 27.8% *vs.* 81.1 ± 16.2%; $p = 0.001$). Regarding antibiotic use (Fig. 3B), there were no significant differences in the number of days in therapy (58.8 ± 32.1 *vs.* 46.7 ± 32.7; $p = 0.31$). Regarding hospital admissions (Fig. 3C), the proportion of patients requiring hospitalization in the past year was greater in the higher DNA concentration group (68.8% *vs.* 35.3%; $p = 0.05$), showing a significant association between DNA level in CF sputum and hospitalization. There was no association between DNA level and chronic PA colonization (Fig. 3D). Similarly, no significant differences were found in pulmonary exacerbations, as 73.3% of patients in the higher DNA concentration group had at least three exacerbations in the past year compared to 41.2% in the lower DNA concentration group ($p = 0.07$). When the absolute values of the number of pulmonary exacerbations in the past 12 months were compared (3.4 ± 1.9 *vs.* 2.4 ± 1.3) there were also no differences ($p = 0.09$). When DNA levels (μg/mL) were compared between genotypes, no differences ($p = 0.449$) were found between F508del homozygous (218.1 ± 96.8), F508del heterozygous (226.2 ± 145.3) and patients with other mutations (300.0 ± 194.9).

4. Discussion

The results of this study demonstrate that extracellular DNA in CF sputum is associated with pulmonary function and hospitalizations. Patients with higher DNA levels showed a significant decrease in FEV_1 and a greater need for hospitalization compared to those with lower DNA levels.

Significantly higher extracellular DNA concentrations are detected in the respiratory secretions of CF patients [14,25]. Extracellular DNA changes the rheological properties of mucus by increasing fluid viscosity and elasticity [12], which is associated with disease severity and airway obstruction [14,19]. Our findings are consistent with those of Marcos et al. [14] in demonstrating a significant correlation between DNA level in CF sputum and pulmonary function. Moreover, patients with higher DNA levels had decreased FEV_1 compared to those with lower DNA levels. Regarding airway colonization, our results have shown no association of DNA levels with the presence of chronic PA, which is also in agreement with previous data [14]. Although the levels of free DNA were previously associated with fungal infection by *Aspergillus fumigatus* [14], the reduced number of patients presenting positive fungal cultures in our sample prevented us from further statistical analysis.

Extracellular DNA levels in CF patients also seem to be associated with hospitalizations. In our study, the higher DNA concentration group had approximately twice as many patients requiring hospitalization compared to the lower DNA concentration group. To our knowledge, this is the first study to demonstrate the effect of DNA levels on pulmonary exacerbations requiring hospitalization and IV antibiotic use. Pulmonary exacerbations are an important predictor of morbidity and mortality in CF [2]. Studies have shown that pulmonary exacerbations contribute to decreased pulmonary function [5,7,8,26], reduced survival [4,5], and impaired quality of life [10]. Because many acute exacerbations require IV antibiotic use and hospital admission, hospitalizations are frequent in CF patients. In our study, 51.1% of patients were hospitalized in the previous year with exacerbations, and 47.1% of them had at least two admissions. Hospitalizations are known to contribute to impaired pulmonary function and increased treatment-related costs [27–29], making monitoring and prevention strategies of great importance in CF management.

Sputum DNA is derived from leukocytes, especially neutrophils, which move into the airways to fight infections [30]. Until recently, extracellular DNA was believed to derive only from cell necrosis and apoptosis [12,25]. However, recent reports showed that neutrophils also release their DNA through a defense mechanism named NETosis, consisting of the release of NETs [31], which are found in large numbers in CF patients [15,16,24]. NETs are formed by extracellular DNA strands bound to histone and granule protein complexes, such as neutrophil elastase and myeloperoxidase [31]. Approximately half of total DNA level in CF sputum is believed to be derived from NETs [15]; although they serve as a “trap” for bacteria and other pathogens, NET excess may contribute to airway obstruction and colonization, in addition to causing damage to lung tissue [32].

Since the 1960s, extracellular DNA levels in mucus have been considered an important contributing factor to lung disease in CF [25], even though the discovery of DNA release through NETs is more recent. Within this context, inhalation therapy with dornase alfa (recombinant human deoxyribonuclease I, rhDNase) has been recognized since the 1990s as part of the treatment of CF patients [33,34]. RhDNase is an enzyme that selectively cleaves extracellular DNA, thus reducing mucus viscosity and favoring mucociliary clearance. Its use is associated with reduced exacerbations and improved pulmonary function and quality of life [35]. In our sample, 93.9% of patients used dornase alfa (Pulmozyme®, Roche), suggesting that even with adequate therapy a high DNA level contributes to CF severity.

Neutrophils express CFTR on their cell surface and on phagolysosomal membranes [32]. Although the different classes of CFTR mutations may exert varying effects on neutrophil dysfunction, our

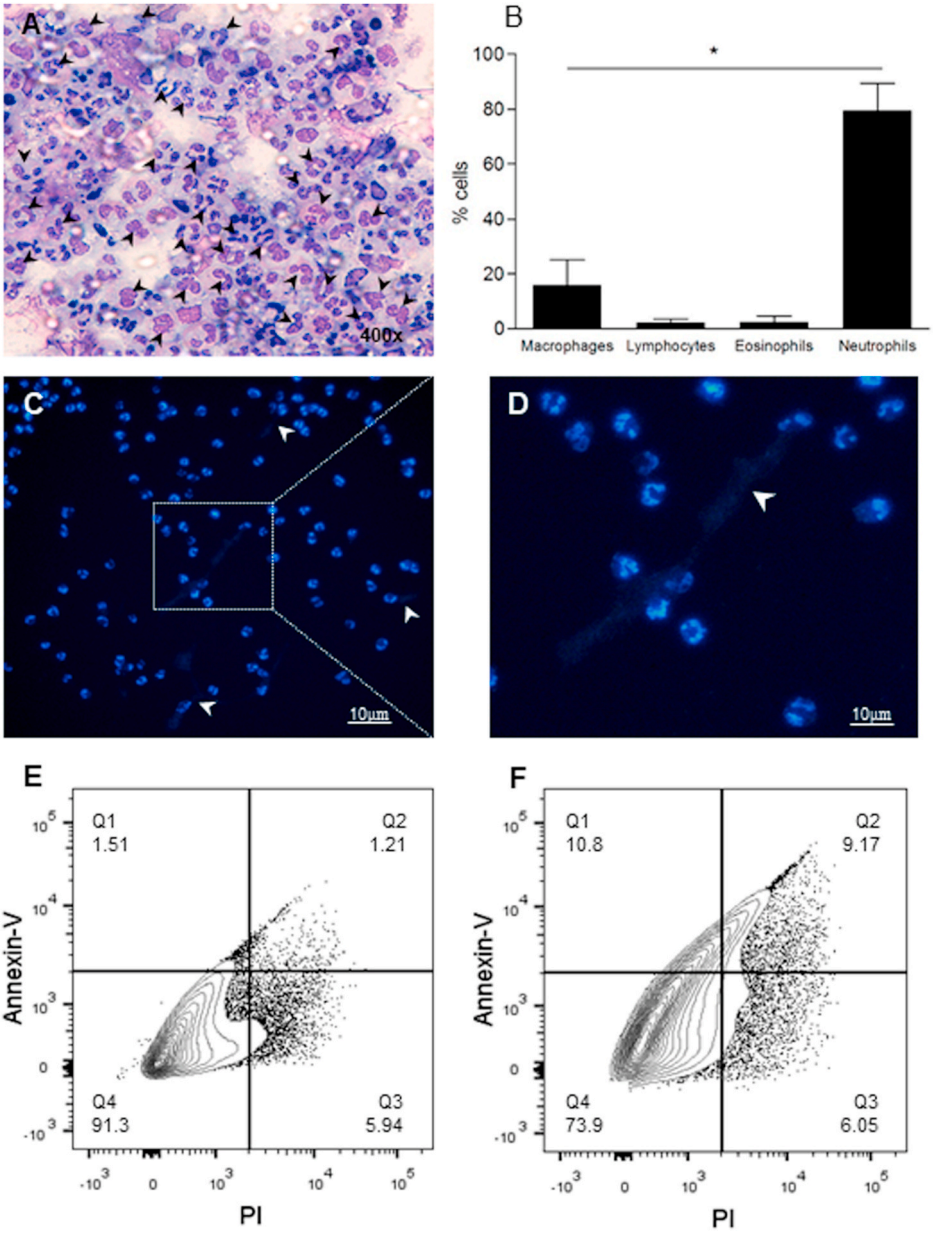

Fig. 1. Cellular profile, extracellular traps and cell death in patients with cystic fibrosis. (A) Representative image of the sputum cellular profile. The arrows indicate the predominance of neutrophils. (B) Differential leukocyte count. The data show the percentage of each cell type, expressed as mean ± standard deviation. (C) Visualization of extracellular DNA networks by immunofluorescence microscopy. Arrows indicate the presence of extracellular DNA fibers (*Hoechst* 1:2000). (D) Magnification of the cells presented in figure (C). (E) and (F) Detection of cell death by flow cytometry with Annexin V and propidium iodide (PI). Representative data from two samples with DNA>243 μg/mL (327 μg/mL and 397.5 μg/mL, respectively), showing a low percentage of apoptosis (Q1), necrosis (Q3) and late apoptosis/necrosis (Q2). Viable cells are shown in the Q4 quadrant. $^{*}p < 0.001$ (ANOVA followed by the *Bonferroni* post-test).

results did no show differences on DNA levels between patients with different genotypes. Recent studies have shown that neutrophils from CF patients have a longer survival because of late apoptosis, which may favor the release of extracellular DNA in the form of NETs [36,37]. This seems to be related to a primary neutrophil defect resulting from a dysfunctional CFTR protein. Thus, the development of target therapies is a potentially effective option in the treatment of CF patients considering the negative consequences of chronic inflammation and NET buildup to the progression of lung disease [32].

Our study has some limitations. We cannot state that the extracellular DNA analyzed here consists exclusively of NETs, as it can also derive from neutrophils and other necrotic or apoptotic leukocytes, although fluorometric quantification is highly objective and recommended in the literature [38] and flow cytometry demonstrated a low rate of cell necrosis and apoptosis [39]. Similarly, although quite likely, we cannot state that the extracellular DNA networks seen in immunofluorescence microscopy effectively are NETs, as such detection requires colocalization studies with specific markers, such as citrullinated histones, myeloperoxidase, or neutrophil elastase [38]. Also, samples were collected only from patients able to produce spontaneously expectorated sputum, without prior induction protocol; therefore, patients with no airway secretion due to milder CF phenotypes were not included. Nonetheless, we included patients with FEV_1 between 21% and 108% of the predicted value, which minimizes potential influences of CF severity on the results.

5. Conclusions

The results show that elevated extracellular DNA levels in CF sputum are associated with reduced pulmonary function and increased hospitalizations. Investigating the mechanisms of NET formation and extracellular DNA release may contribute to a better understanding and development of specific therapies.

CRediT authorship contribution statement

Taila Cristina Piva: Conceptualization, Investigation, Formal analysis, Writing - original draft, preparation. **Carolina Luft:** Investigation,

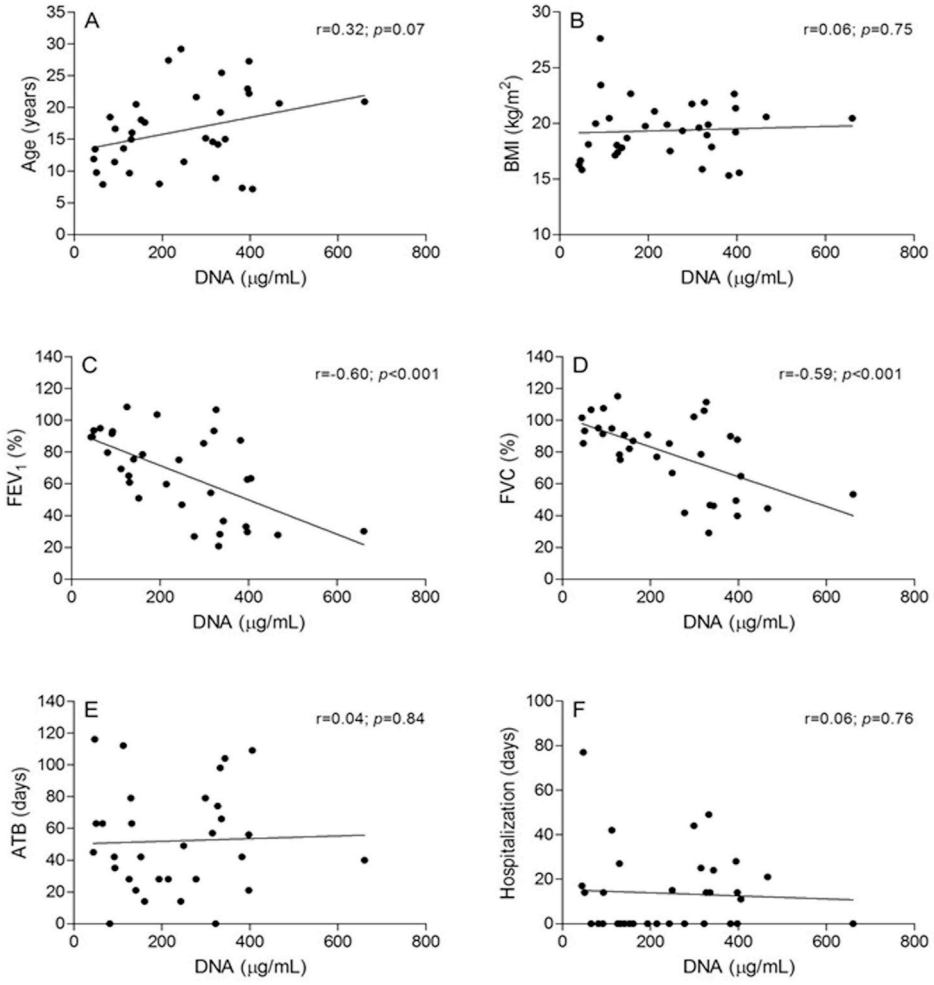

Fig. 2. Correlation between the amount of extracellular DNA in the sputum with (A) age; (B) body mass index - BMI; (C) forced expiratory volume in 1 s – FEV_1; (D) forced vital capacity - FVC; (E) total number of days of oral and intravenous antibiotic use in the previous 12 months; (F) total number of days of hospitalization in the previous 12 months. Coefficients of correlation (r) and significance (*p*) evaluated using Pearson or Spearman tests.

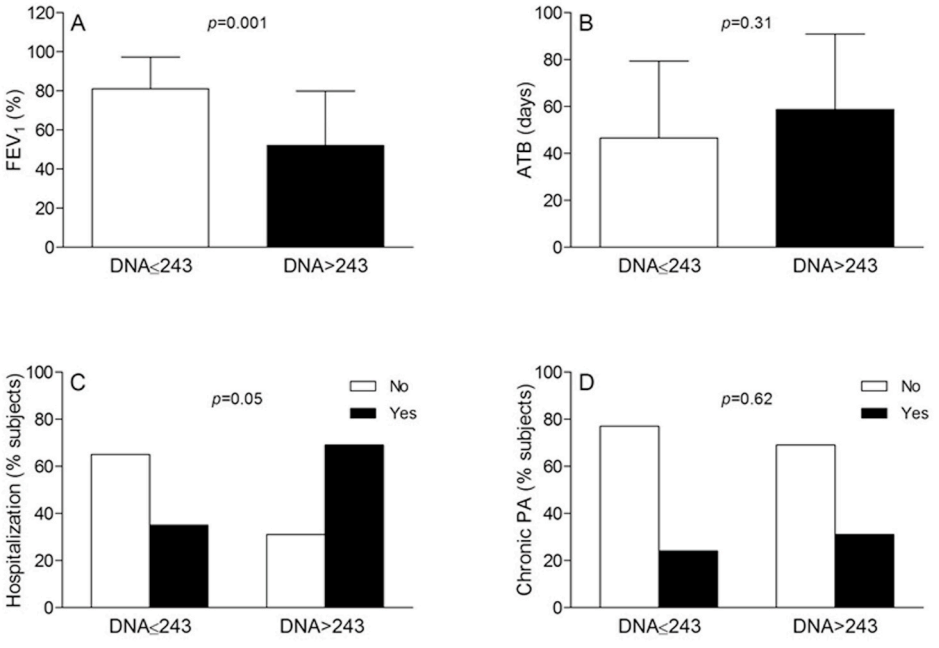

Fig. 3. Comparison of (A) lung function (forced expiratory volume in 1 s – FEV_1) and (B) total days of antibiotic use in the previous 12 months, according to the amount of extracellular DNA in the sputum (≤243.0 μg/mL *vs.* >243.0 μg/mL). A *t*-test for independent samples was used for comparisons and results are presented as mean and standard deviation. Significance set at $p < 0.05$. (C) and (D) shows, respectively, the association of the hospitalization frequency and chronic *Pseudomonas aeruginosa* (PA) infection according to the amount of DNA in the sputum. A Pearson Chi-square test was used for analysis and results are presented as relative frequency. Significance set at $p < 0.05$.

Writing - review & editing. **Krist Helen Antunes:** Investigation, Writing - review & editing. **Paulo José Cauduro Marostica:** Supervision, Writing - review & editing. **Leonardo Araújo Pinto:** Supervision, Writing - review & editing. **Márcio Vinícius Fagundes Donadio:** Conceptualization, Supervision, Formal analysis, Writing - original draft, preparation.

Declaration of competing interest

The authors declare that they have no known competing financial interests or personal relationships that could have appeared to influence the work reported in this paper.

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Appendix A. Supplementary data

Supplementary data to this article can be found online at https://doi.org/10.1016/j.rmed.2020.106144.

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