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Aerobic fitness is associated with extracellular DNA levels in the sputum of patients with cystic fibrosis

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Abstract

Aims: Patients with cystic fibrosis (CF) develop with progressive loss of lung function and aerobic fitness. However, the precise mechanisms of exercise intolerance are still controversial and appear to be influenced by several factors. This study aimed to evaluate the association of aerobic fitness with free DNA levels in the sputum of patients with CF.

Methods: This cross-sectional study included patients with CF older than 6 years, free from active exacerbations, but who were able to produce spontaneously expectorated sputum. Extracellular DNA in the sputum was quantified. Lung function (spirometry) and aerobic fitness (cardiopulmonary exercise testing [CPET]) were performed. In addition, demographic, anthropometric and clinical data were collected.

Results: Sixteen patients with a mean age of 19.4 ± 6.9 years and mean forced expiratory volume in the first second (FEV_1) of 51.8 ± 28.1 (% of predicted) were included. Mean peak oxygen consumption (VO_2peak) was 32.8 ± 5.2 mL• kg^{-1}• min^{-1}, oxygen saturation at the end of the test was 90.6% ± 6.3% and mean extracellular DNA levels was 305.3 ± 153.6 μg/mL. Individuals with a VO_2peak ≤ 30 mL• kg^{-1}• min^{-1} ($P = .03$) and a SpO_2 ≤ 90% at the end of the test ($P = .03$) had a greater amount of extracellular DNA in the sputum. The proportion of patients with reduced VO_2peak in the group of patients with the lowest concentration of DNA in the sputum (<243 μg/mL) was significantly lower (0% vs 100%; $P = .04$).

Conclusion: There is an association between the presence of free DNA in sputum and aerobic fitness in patients with CF.

1 | INTRODUCTION

Cystic fibrosis (CF) is a genetic disease that affects multiple organs and systems. It is characterized by a progressive decline in pulmonary function and by the presence of exercise intolerance, which is associated with airway obstruction and the presence of chronic inflammation and recurrent infections.[1,2]

Elevated levels of extracellular DNA, mainly originating from neutrophil extracellular traps (NETs), have been described in both bronchoalveolar lavage and sputum of CF patients.[3-5] The presence of DNA changes the viscosity and elasticity of the mucus, contributing to airway obstruction. In addition to this, the presence of chronic airway neutrophilic inflammation is considered an important factor for the early development of lung disease.[6]

DNA levels in airway fluid samples from individuals with CF are already known to negatively correlate with lung function.[7] This suggests that the DNA found in airway fluids may contribute to lung disease from the beginning of life. A recent study showed that subjects with levels of DNA in the sputum greater than 243 μg/mL had a significant decrease in forced expiratory volume in the first second (FEV_1) and greater need for hospitalisation.[8]

Despite the evidence that demonstrates the influence of sputum DNA levels on clinical and morbidity markers, the association of free DNA levels in sputum with aerobic fitness in subjects with CF is unknown to date. Studies demonstrate an association of aerobic fitness, assessed through peak oxygen consumption (VO_2peak), with mortality in patients with CF.[9] Thus, it is clinically relevant to investigate factors that may be associated with exercise capacity in these individuals. For that, the aim of the present study was to evaluate the association of aerobic fitness, assessed through cardiopulmonary exercise testing (CPET), with extracellular DNA levels in the sputum of patients with CF. Our hypothesis is that patients with lower levels of free DNA in sputum present better aerobic capacity.

What's known

- The precise mechanisms of exercise intolerance in patients with cystic fibrosis are still controversial, although several factors have been associated, including sex, age, nutrition, airway chronic colonisation, pulmonary function, genotyping and respiratory muscle function.
- Although the presence of increased sputum production in subjects with cystic fibrosis is well-known, the association of elevated extracellular DNA levels in the sputum with aerobic fitness is still unknown.

What's new

- High levels of extracellular DNA in the sputum of patients with cystic fibrosis are associated with reduced aerobic fitness.
- A better understanding of the factors that contribute to exercise intolerance in cystic fibrosis is fundamental in the search for more efficient prevention and treatment strategies.

2 | METHODS

This is a cross-sectional study. Subjects with genetic diagnosis of CF, not under cystic fibrosis transmembrane conductance regulator modulator therapy, aged ≥ 6 years, who underwent regular monitoring at the CF outpatient clinic of Hospital São Lucas (PUCRS), and who were able to produce spontaneous sputum expectoration were included. Subjects with osteo-articular and musculoskeletal disorders that would interfere with CPET performance were excluded. In addition, individuals with inadequate sputum samples, who were under antibiotics use or presented with any sign of pulmonary exacerbation in the last 15 days, including fever, increased cough and sputum production, were excluded.[10] The sample was selected by convenience and data were collected from August 2018 to May 2019.

Considering that there are no previous studies that evaluated the association between free DNA levels in the sputum and aerobic fitness, we used lung function data to estimate the necessary sample size.[8] Considering an expected correlation between variables from 0.5 to 0.7, a significance level of 0.05, and a power of 80%, an approximate sample size of 15 subjects was estimated. This study was approved by the Ethics Committee of Pontifícia Universidade Católica do Rio Grande Do Sul (CAAE: 91277118.5.0000.5336). All legal guardians and subjects over 18 years old signed an informed consent form. In addition, children and adolescents up to 18 years old signed an assent form.

Patients with a history of sputum expectoration, identified through the follow-up forms and medical records, were selected for inclusion in the study. Sputum collection and spirometry were performed during routine outpatient visits. Afterwards, patients were referred to the Pediatric Physical Activity Laboratory (Centro Infant, PUCRS) to perform CPET. In addition, clinical and genetic data were collected, including the presence of pancreatic insufficiency, chronic colonisation by *Pseudomonas aeruginosa* (PA) and the type of genetic mutation. Chronic colonisation by PA was defined as positive cultures in more than 50% of the samples in the 12 months prior to analysis.[11]

For sputum collection, subjects who used dornase alfa (Pulmozyme®, Roche) were recommended to wait at least 12 hours between nebulisation and the collection procedure, because of the drug's action on DNA. As previously described,[8] in order to avoid contamination of the sample, subjects were instructed to clean the mouth beforehand to remove the superficial bacterial flora. After, participants were asked to spontaneously expectorate in a sterile container. The samples were kept in a refrigerated environment for up to 2 hours until processing and analysis.

2.1 | Anthropometric data

Anthropometric measurements were performed as previously described.[12,13] Briefly, weight and height were measured in orthostasis using a digital scale (G-tech, Glass 1 FW, Rio de Janeiro, Brazil) and a portable stadiometer (AlturaExata, TBW, São Paulo, Brazil), respectively. The absolute body mass index (BMI) was calculated and expressed as kg/m^2.

2.2 | Lung function

Lung function was evaluated through spirometry, following the recommendations of the American Thoracic Society – European Respiratory Society ATS/ERS.[14] The tests were performed with subjects in orthostasis using the KOKO spirometer (Louisville, CO, USA), as previously reported.[13] The main variables evaluated included forced vital capacity (FVC), FEV_1 and forced expiratory flow from 25% to 75% of vital capacity ($FEF_{25\%-75\%}$). The data were expressed as a percentage of the predicted based on the reference equations from the Global Lung Function Initiative.[15]

2.3 | Quantification of extracellular DNA

Sputum processing was performed at the Pediatric Respirology Laboratory (Centro Infant, PUCRS), as previously described.[8] In brief, the sample was homogenized in dithiothreitol (DTT) and Dulbecco's phosphate-buffered saline (DPBS), followed by the filtration and centrifugation process. For DNA analysis, the supernatant was precipitated in absolute ethanol and 3M sodium acetate and incubated overnight at −20°C. The sample was centrifuged and the pellet was resuspended in 70% ethanol, a new centrifugation process was performed and the new pellet was diluted in nuclease-free water for DNA quantification. DNA was quantified using the Qubit 2.0 fluorometer (Invitrogen) using the dsDNA HS test kit (Invitrogen), following the manufacturer's guidelines.

2.4 | Cardiopulmonary exercise testing

Aerobic fitness was evaluated through CPET, following the recommendations of the American Thoracic Society and the American College of Chest Physicians.[16] Detailed protocol has been previously described.[17] A computerized system (Aerograph, AeroSport®, United States), coupled to a gas analyser (VO2000, MedGraphics®, United States) and a treadmill (KT-10400, Inbramed®, Brazil) were used. Briefly, an adapted ramp protocol[18] was used and participants were instructed to walk for 2 minutes to adapt to the treadmill, at a speed of 3 km/h, without inclination. Afterwards, there were increments in the speed of 0.5 km/h every minute, with a fixed slope of 3% until the end of the test.[17] All subjects were encouraged to keep pace until exhaustion or the appearance of limiting signs and/or symptoms (dyspnoea, pain in the legs and/or dizziness). The test was considered as a maximum when at least three of the following criteria was observed: exhaustion or inability to maintain the required speed, RER > 1.05, HRmax > 85% of the HR estimated (208 − 0.7 × age[19]), and the presence of a plateau in VO_2.[20,21] The main variables evaluated included VO_2peak, carbon dioxide production (VCO_2), minute ventilation (V_E), respiratory exchange rate (RER), ventilatory equivalents for oxygen consumption (V_E/VO_2) and for carbon dioxide production (V_E/VCO_2), peripheral oxygen saturation (SpO_2) and maximum heart rate (HRmax). The anaerobic threshold (AT) was determined using the criteria of an increase in V_E/VO_2 with no correspondent increase in V_E/VCO_2. Breathing reserve was calculated as the difference between the estimated maximum voluntary ventilation (MVV)[22] at rest and the peak V_E, and was reported as a percentage of MVV. At the beginning and end of the test, the subjective perception of dyspnea and leg discomfort were evaluated using the modified Borg scale.

2.5 | Statistical analysis

In order to assess the distribution of variables, the Shapiro–Wilk test was used. Continuous variables were presented as mean and standard deviation. Categorical variables were presented in absolute and relative frequencies. Correlations between extracellular DNA levels in sputum and aerobic fitness variables were performed using Pearson's linear correlation test. For comparisons, patients were divided according to VO_2peak (≤30 mL• kg^{-1}• min^{-1}), SpO_2 at the end of the test (≤90%) and levels of extracellular DNA in the sputum (<243 μg/mL).[8] The Student's *t* test for independent samples and Pearson's chi-square test were used. SPSS version 18.0 (SPSS Inc., USA) was used for all the analysis. The level of significance adopted was $P < .05$.

3 | RESULTS

Sixteen patients (62.5% males) with a mean age of 19.4 ± 6.9 years were included (Figure S1). Fifty per cent were heterozygous for the ΔF508 mutation. The mean extracellular DNA levels in sputum were 305.3 ± 153.6 μg/mL. Mean time between last Pulmozyme inhalation and sputum collection was 19.1 ± 9.6 hours and no significant correlation with DNA levels was found ($r = .06$; $P = .827$). Regarding lung function, the mean (% of predicted) FEV_1 was 51.8 ± 28.1 and FVC was 65.1 ± 25.4, which indicates a sample with moderate lung function impairment. The characterisation data of the included sample is presented in Table 1.

The physiological responses found on CPET are shown in Table 2. VO_2peak was 32.8 ± 5.2 mL• kg^{-1}• min^{-1}, SpO_2 at the end of the test was 90.6% ± 6.3% and HRmax was 178.9 ± 14.0 beats•minute. When correlating the levels of extracellular DNA in the sputum with CPET variables, we found a negative and moderate correlation with SpO_2 at the end of the test ($r = -.58$; $P = .02$).

Figure 1 shows the comparison of extracellular DNA levels in sputum between patients with VO_2peak ≤ 30 mL• kg^{-1}• min^{-1} and VO_2peak > 30 mL• kg^{-1}• min^{-1} (Figure 1A), as well as the comparison between patients with SpO_2 ≤ 90% and SpO_2 > 90% (Figure 2B). The results demonstrated that subjects with VO_2peak ≤ 30 mL• kg^{-1}• min^{-1} ($P = .03$) and SpO_2 ≤ 90% at the end of the test ($P = .03$) had greater levels of extracellular DNA in the sputum. No difference was found between patients with or without chronic colonisation by PA ($P = .25$).

When assessing the association between DNA levels in the sputum and the reduction in VO_2peak (Figure 2), the results demonstrated that the proportion of patients with reduced VO_2peak in the group with the lowest DNA concentration in the sputum (< 243 μg/mL) was significantly lower (0% vs 100%; $P = .04$), indicating an association between the presence of free DNA and aerobic fitness.

4 | DISCUSSION

The data presented here have shown that there is an association between the presence of free DNA in sputum and aerobic fitness in patients with CF. Individuals with VO_2peak ≤ 30 mL• kg^{-1}• min^{-1} and SpO_2 ≤ 90% at the end of CPET presented higher levels of

TABLE 1 Characteristics of the study sample

Variables	n = 16
Demographic	
Age (years)	19.4 ± 6.9
Male, n (%)	10 (62.5)
Anthropometric	
Weight (kg)	51.3 ± 14.9
Height (cm)	158.9 ± 19.1
BMI (kg/m^2)	19.7 ± 2.8
Clinical	
Pancreatic insufficiency, n (%)	14 (87.5)
Chronic *Pseudomonas aeruginosa*, n (%)	5 (31.3)
Use of Dornase alfa, n (%)	16 (100)
Extracellular DNA in sputum (μg/mL)	305.3 ± 153.6
Genotyping	
F508del homozygous, n (%)	3 (18.8)
F508del heterozygous, n (%)	8 (50.0)
Other mutations, n (%)	5 (31.3)
Lung function	
FEV_1 (% predicted)	51.8 ± 28.1
FVC (% predicted)	65.1 ± 25.4
FEV_1/FVC (% predicted)	75.4 ± 13.8
$FEF_{25\%-75\%}$ (% predicted)	32.6 ± 29.2

Abbreviations: BMI, body mass index; $FEF_{25\%-75\%}$, forced expiratory flow from 25% to 75% of vital capacity; FEV_1, forced expiratory volume in 1 second; FVC, forced vital capacity.

Values expressed as absolute frequency (relative) or mean ± standard deviation.

TABLE 2 Cardiopulmonary exercise testing variables

Variables evaluated	n = 16
Resting	
HR (beats•min)	92.1 ± 22.0
SpO_2 (%)	96.4 ± 1.9
VO_2 (L•min)	0.4 ± 0.2
VO_2 (mL• kg^{-1}• min^{-1})	8.2 ± 3.0
Borg for dyspnea	0.3 ± 0.5
Borg for leg discomfort	0.9 ± 2.0
Anaerobic threshold	
HR (beats/min)	147.3 ± 27.5
SpO_2 (%)	93.1 ± 4.7
VO_2 (L•min)	1.2 ± 0.4
VO_2 (mL• kg^{-1}• min^{-1})	23.3 ± 5.5
VO_2 (%)	71.5 ± 14.3
V_E (L•min)	27.1 ± 9.5
V_E/VO_2	24.1 ± 3.3
V_E/VCO_2	23.7 ± 3.1
Peak exercise	
HR (beats•min)	178.9 ± 14.0
SpO_2 (%)	90.6 ± 6.3
VO_2 (L•min)	1.7 ± 0.7
VO_2 (mL• kg^{-1}• min^{-1})	32.8 ± 5.2
V_E (L•min)	44.2 ± 15.9
V_E/VO_2	28.2 ± 5.3
V_E/VCO_2	24.5 ± 3.8
RER	1.1 ± 0.1
Breathing reserve (%)	36.8 ± 19.9
Borg for dyspnea	3.6 ± 2.9
Borg for leg discomfort	3.7 ± 2.2

Abbreviations: HR, heart rate; RER, respiratory exchange ratio; SpO_2, peripheral oxygen saturation; V_E, minute ventilation; V_E/VCO_2, ventilatory equivalent ratio for carbon dioxide production; V_E/VO_2, ventilatory equivalent ratio for oxygen uptake; VO_2, oxygen uptake.

Values expressed as mean ± standard deviation.

extracellular DNA in the sputum. A better understanding of the factors that contribute to exercise intolerance in CF is fundamental in the search for more efficient prevention and treatment strategies.

The multisystemic characteristic and the evolution of the disease often culminate in the development of some degree of intolerance to physical exercise in patients with CF.[23] However, the precise mechanisms of exercise intolerance are still controversial, as evidence has shown that multiple elements (sex, age, nutrition, airway chronic colonisation, pulmonary function, genotyping, respiratory muscle function) may be involved.[24-27] Moreover, Sovtic et al[28] demonstrated that static hyperinflation (expressed as RV/TLC) is associated with decreased exercise capacity in children with CF. In addition to these factors, levels of extracellular DNA in sputum should also be considered as a possible determinant of exercise capacity. Although the mechanism of DNA release by neutrophils is considered as a defence mechanism,[29] the presence of increased levels of free DNA in sputum is associated with worse clinical conditions, lung function and the presence of infections,[8] which may also contribute to the reduction of aerobic fitness.

Nevertheless, evidence demonstrates an association of VO_2peak, measured by CPET, with mortality in CF. Patients with lower VO_2peak (<45 mL• kg^{-1}• min^{-1} or 82% of predicted) were described as having a 4.9 times greater risk of a fatal outcome, highlighting the evaluation of exercise capacity as an important tool for prognosis.[9] In addition, a study showed an association of VO_2peak with the risk of hospitalisation in subjects with CF,[30] emphasising the importance of periodic assessment of aerobic fitness. According to CF guidelines of care, the assessment of aerobic fitness should be performed annually, aiming to identify the main mechanisms behind exercise intolerance.[31] To the best of our knowledge, this is the first study that evaluated the association of free DNA levels in sputum with aerobic fitness in patients with CF. Our results suggest that greater amounts of DNA in sputum are associated with reduced VO_2peak and, consequently, mortality, considering that patients included in our study had a mean VO_2peak of 32.8 ± 5.2 mL• kg^{-1}• min^{-1}.

The inflammatory response contributes to the development of lung disease in CF. Neutrophils accumulate in the airways in response

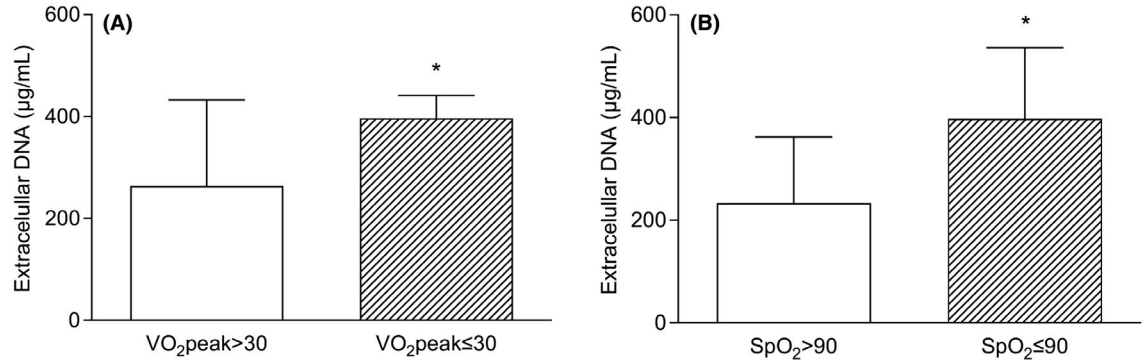

FIGURE 1 Comparison of extracellular DNA levels in the sputum according to peak oxygen consumption (VO_2peak) > 30 mL• kg^{-1}• min^{-1} or ≤ 30 mL• kg^{-1}• min^{-1} (A) and peripheral oxygen saturation (SpO_2) at the end of the test >90% or ≤90% (B) *$P < .05$

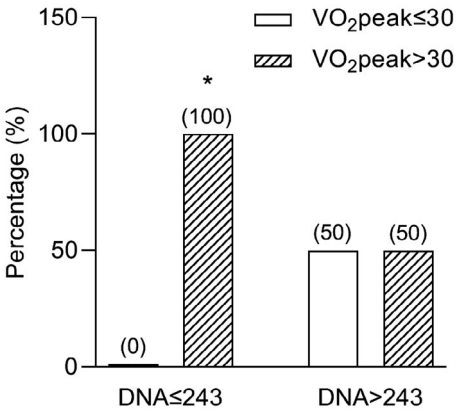

FIGURE 2 Comparison of the percentage of subjects with extracellular DNA levels in the sputum ≤243 µg/mL or >243 µg/mL and peak oxygen consumption (VO_2peak) > 30 mL• kg^{-1}• min^{-1} or ≤ 30 mL• kg^{-1}• min^{-1}. *$P < .05$

to frequent infections and inflammation, resulting in chronic neutrophilic inflammation[32] and direct damaging of the airway epithelium.[33] Significantly high concentrations of mucins and inflammatory markers are observed in the bronchoalveolar lavage of children with CF, despite the low incidence of pathogen and absence of structural lung injury in the first years of life, suggesting that the early accumulation of mucus and inflammation are primarily responsible by the development of early lung disease.[6] Our results have shown no differences in DNA levels between patients with or without chronic colonisation by PA. Nevertheless, evidence demonstrates the association of DNA levels in sputum with clinical and morbidity markers in patients with CF. Studies show that DNA levels in airway fluid samples negatively correlate with lung function.[5,7,8] Extracellular DNA, originated mainly from neutrophils recruited into the lungs, changes the mucus rheological properties, altering its viscosity and elasticity, which is associated with disease severity and airflow obstruction.[5] Our results demonstrate that subjects with SpO_2 ≤ 90% at the end of CPET present greater levels of extracellular DNA in the sputum. Patients with CF may develop exercise-induced hypoxemia, which could lead to a reduction in aerobic fitness.[34] Evidence shows that performing airway clearance prior to exercise may alter ventilatory mechanics and increase ventilatory efficiency during exercise,[35] which may also be, at least in part, related to the removal of extracellular DNA from airways. In addition, patients with more severe lung disease experience a greater decline in SpO_2 levels during maximal exercise.[36] SpO_2 is an important clinical marker of exercise intolerance and its possible indirect association with levels of free DNA in sputum allows us to advance in the understanding of the mechanisms that may be associated with desaturation in these subjects.

Our study presents limitations, including the fact that samples for quantification of extracellular DNA were collected only from individuals capable of spontaneously expectorating, without any previous induction protocol. Thus, patients who did not present airway secretion, which may include mild phenotypes, were not included in the study. In addition, the small sample size could also be considered as a study limitation, as it prevented us from performing further multivariate analyses.

5 | CONCLUSIONS

The results of the present study show that high levels of extracellular DNA in the sputum of patients with CF are associated with reduced aerobic fitness. A better understanding of how these mechanisms affect exercise tolerance is important for the development of prevention and treatment strategies in the management of patients with CF.

DATA AVAILABILITY STATEMENT

The data that support the findings of this study are available from the corresponding author upon reasonable request.

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DISCLOSURES

The authors have no conflict of interests to declare.

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SUPPORTING INFORMATION

Additional Supporting Information may be found online in the Supporting Information section.

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