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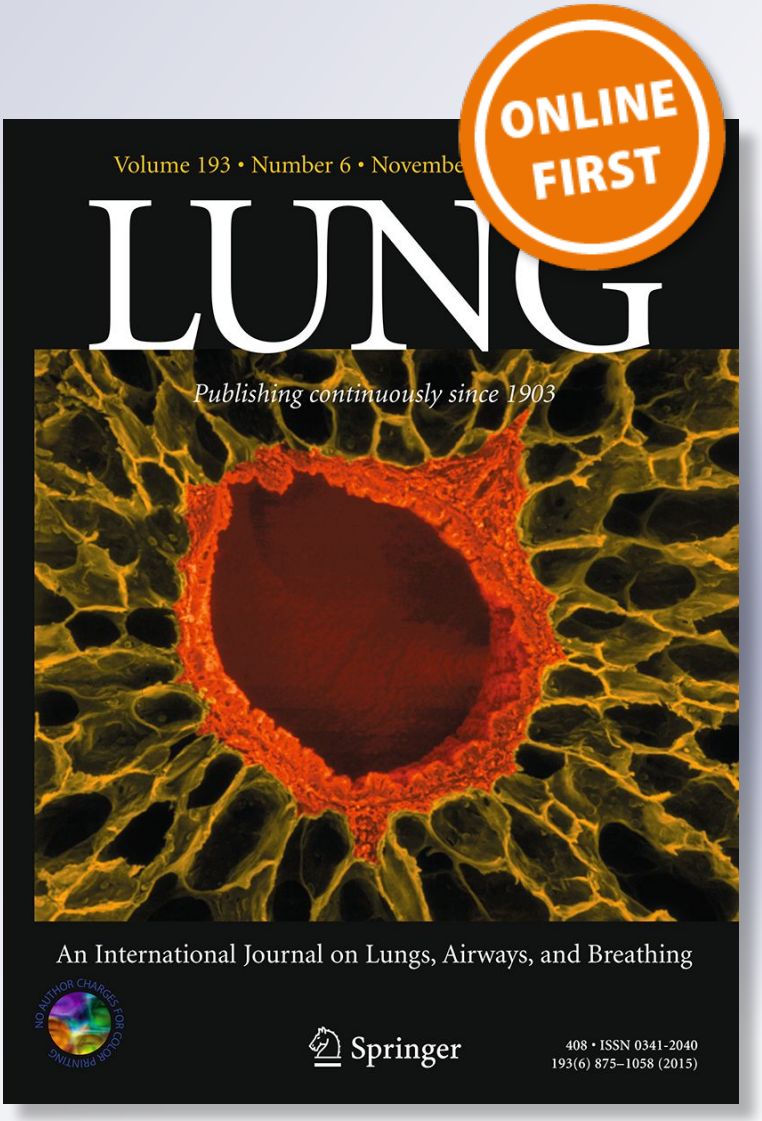

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CYSTIC FIBROSIS

Chloride Conductance, Nasal Potential Difference and Cystic Fibrosis Pathophysiology

Elenara da Fonseca Andrade Procianoy[1,2] · Fernando Antônio de Abreu e Silva[1,2] · Paulo José Cauduro Maróstica[1,3] · Paul M. Quinton[4]

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Abstract

Purpose Cystic fibrosis (CF) is a multisystem genetic disease caused by dysfunction of the epithelial anionic channel Cystic Fibrosis Transmembrane conductance Regulator (CFTR). Decreased mucociliary clearance because of thickened mucus is part of the pulmonary disease pathophysiology. It is controversial if the thickened airway surface liquid (ASL) is caused by the deficient chloride secretion and excessive sodium (through ENaC) and water hyperabsorption from the periciliar fluid or by the lack of bicarbonate secretion with relative acidification of the ASL. Correlations between the magnitude of in vivo chloride conductance with phenotypic characteristics and CF genotype can help to elucidate these mechanisms and direct to new treatments.

Methods Nasal potential difference was measured in 28 CF patients (age from 0.3 to 28 year) and correlated with pulmonary function, pancreatic phenotype, pulmonary colonization and genotype severity.

Results The CFTR-chloride conductance was better in older patients ($r = 0.40$; $P = 0.03$), in patients with better pulmonary function ($r = 0.48$; $P = 0.01$), and was associated with genotype severity. Higher chloride diffusion in the presence of a favorable chemical gradient was associated with *Pseudomonas aeruginosa* negativity ($P < 0.05$). More negative NPDmax was associated with pancreatic insufficiency ($P < 0.01$) as well with genotype severity, but not with the pulmonary function.

Conclusions The anion permeability through CFTR, mainly chloride, but bicarbonate as well, is the most critical factor in CF airway pathophysiology. Treatments primarily directed to correct CFTR function and/or airway acidity are clearly a priority.

Keywords Cystic fibrosis · Genetic disease · CFTR · Bicarbonate · *Pseudomonas aeruginosa*

Introduction

Cystic fibrosis (CF) is a recessive genetic disease caused by CF transmembrane conductance regulator (CFTR) gene mutations that encodes the CFTR protein [1]. CFTR protein is an epithelial ion channel that transports chloride (Cl^-) and bicarbonate (HCO_3^-), and regulates other ion channels (namely sodium, potassium, other chloride and calcium channels). It also interacts with other membrane proteins to maintain epithelial tight-junctions and barriers to fluid flow. CFTR protein adjusts the levels of acidity in secretions that, along with fluid and chloride abnormalities, lead to some of the common manifestations of the CF clinical syndrome such as small airway disease and pancreatic insufficiency [2]. The disrupted ion transport also causes cell hyperpolarization or depolarization depending on whether the cell is secretory or absorptive, respectively [3].

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Impairment of the mucociliary clearance (MCC) because of increased viscosity of the airway surface liquid (ASL) is thought to predispose CF patients to small airway disease with chronic airway infection, associated airway inflammation, progressive airway obstruction and lung injury [3–6]. How the dysfunctional CFTR leads to increased viscosity of ASL remains somewhat controversial since some researches hold that it is caused by deficient chloride secretion and excessive sodium and water reabsorption from the periciliar fluid [6] while other hold that it is caused by a lack of bicarbonate secretion with relative acidification of airway surface liquids [4].

Nasal potential difference (NPD) estimates the relative ion conductance based on transepithelial voltage (Vt) changes across nasal surface epithelial cells in vivo. The loss of CFTR-dependent anion conductance causes characteristic hyperpolarization, which is associated with a characteristic pattern of Vt changes in response to specific transport inhibitors and agonists [7, 8]. Recently, US and European NPD protocols were published with new and better quantitative and discriminatory parameters for using the NPD as a biomarker of CFTR function [9, 10].

In this study, we measured the NPD to correlate the in vivo CFTR-chloride transport in the nasal epithelium with CF pulmonary function and airway bacterial colonization. Secondarily, we analyzed the correlation with pancreatic phenotype, nutritional status, and genotype. Our results may help in further understanding of how abnormal anion transport is related to CF airway disease pathophysiology and help contribute to faster development of new and more effective treatments.

Methods

Study Subjects

CF patients in a clinically stable condition were recruited from the pediatric and adult CF Clinics of Hospital de Clínicas de Porto Alegre (HCPA) [1]. Subjects or their parents gave and signed a written informed consent. The study was reviewed and approved by the Ethical Review Board of HCPA and registered at HCPA Post-Graduation Research Group as number 05-125.

Data regarding age, sex and sweat chloride were collected from medical records. The pulmonary function was assessed as the percentage of predicted normal forced expiratory volume in one second ($ppFEV_1$) and as the FEV_1 z-score for age, sex and height for each individual subject measured by spirometry using the global lung function 2012 equation of the Global Lung Function Initiative [11]. Lung infection was defined as a single positive respiratory tract culture (throat, sputum, or bronchoalveolar lavage culture) within the previous six months for *Pseudomonas aeruginosa* (Pa) regardless of whether the subject cultured positive for other organisms. The pancreatic status (pancreatic insufficient (PI) or pancreatic sufficient (PS)) was evaluated based on need for pancreatic enzyme replacement therapy and/or on fecal elastase measurement. The nutritional status was assessed by the absolute body mass index (BMI) and BMI-for-age z-score. For patients older than 18 years of age, the BMI-for-age z-score of 18-year-old subjects was used as a reference.

CFTR genotype data included analyzes by Real Time-PCR of the 10 most frequent CFTR mutations in South Brazil (F508del, R347P, R347H, R334W, G542X, G551D, R553X, S549N, W1282X and N1303K) [12]. DNA sequencing was done in some patients if the PCR was negative. Each genotype was assigned to one of three groups according to the CFTR mutation, regardless of clinical features. The "severe" genotype group included patients with two mutations of class I, II, or III; the "mild" genotype group included patients with at least one mutation of class IV, V or VI, and the "unknown" genotype group included patients with no identified CFTR mutation or without an available CFTR genetic test. The CFTR mutations were assigned to classes based on the list provided by Elborn [1] and completed according to the genotype severity.

Nasal Potential Difference

NPD measurements were performed as previously described using the dermal abrasion NPD-method [13] under standardized conditions. After direct rhinoscopy to assess nasal epithelium integrity, absence of polyps, and nasal turbinate anatomy the most stable voltage measure under the inferior nasal turbinate after basal solution perfusion was recorded as the maximum NPD (NPD-max). Other NPD measurements were recorded at the end of 3 sequential phases of perfusion of each test solution as follows: (1) basal solution plus 100 µM amiloride (2) low-chloride plus 100 µM amiloride solution, and (3) low-chloride plus 100 µM amiloride plus 10 µM isoproterenol solution.

The following changes in NPD were recorded in response to: (1) amiloride (Δ-amil), which gives the response to blocking Na+ uptake through ENaC; (2) low-chloride plus amiloride solution (Δ-ØCl), which gives the Vt in response to a chloride gradient; (3) low-chloride plus amiloride plus isoproterenol solution (Δ-iso), which gives the Vt induced by isoproterenol via cAMP-mediated chloride conductance through CFTR. The sum of Δ-ØCl + Δ-iso was equivalent to the total chloride response (TCR). TCR was taken as the most sensitive and specific indicator of the CFTR-dependent chloride conductance, and therefore, as the activity of CFTR-chloride channel [14].

Statistical Analysis

Descriptive analyzes of categorical variables were presented as numerical and percentage (*n* (%)) values and of continuous variables as mean ± standard deviation (SD). Correlations were explored using Pearson's rank correlation or Chi-Square coefficient, as appropriate. Comparisons between groups were tested by the *T*-test, nonparametric Mann–Whitney test or by ANOVA post-hoc multiple comparisons.

Analyzes were performed using SPSS 18.0 software. All *P* values were two-sided and considered significant if $P < 0.05$.

Results

The NPD was initially measured in 29 CF patients. One patient was excluded because the CF diagnosis was questioned. Another patient with a rare genotype (5 T–5 T) was retained because she demonstrates a classical phenotype including bronchiectasis, pancreatic insufficiency and sweat chloride > 60 mmol/L. The demographic data, the CFTR mutations, and NPD parameters of the CF patients are presented in Table 1. The NPD measurements according to pancreatic phenotype, *Pseudomonas aeruginosa* colonization, and genotype severity are presented in Table 2.

All patients demonstrated typical CF-NPD tracings with more negative NPD-max (− 35.14 ± 11.92 mV), raised Δ-amil (Δ-amil: 24.46 ± 10.38) and reduced TCR (TCR: − 1.82 ± 4.15) compared to our reference NPD parameters in normal controls (NPD-max − 15.00 ± 4.25 mV, Δ-amil: 6.35 ± 3.5 and TCR: 11.82 ± 3.05). The TCR ranged from 6 to − 10 mV and showed correlation with the age, being higher in older patients ($r = 0.40$; $P = 0.03$). No correlation was found between all NPD parameters and sweat chloride.

The TCR was also correlated with pulmonary phenotype. Better ppFEV1 and FEV_{1-z} score were significantly correlated with a larger TCR ($r = 0.48$; $P = 0.01$ and $r = 0.49$; $P < 0.01$, respectively). However, after adjusting for age, the correlation between TCR and pulmonary function approached the limit of significance ($r = 0.38$; $P = 0.05$), which may be due to the small sample size. CFTR function was apparently not associated with Pa colonization. But Pa colonization appeared to be associated with Δ-ØCl: A more negative Δ-ØCl (− 2.50 mV) was observed in Pa-negative patients compared to Pa-positive patients (Δ-ØCl = 0.56) ($P < 0.05$). Neither was CFTR function associated with pancreatic phenotype, since both PS and PI patients showed residual CFTR function. No association was found between TCR and nutritional status.

The TCR appeared to reflect genotype severity, being smallest in the severe genotype group (− 0.80 ± 3.80), intermediately reduced in the unknown genotype group (− 2.43 ± 4.89) and largest in the mild genotype group (− 3.67 ± 3.04). However, the differences between the groups did not attain statistical significance in this small population.

NPD-max and the Δ-amil were not correlated with pulmonary function. Neither did they associate with airway Pa colonization. However, NPD-max appeared to reflect

Table 1 Clinical data, genotype and NPD parameters of CF patients

Parameter	Values
Age (years)	15.47 ± 6.09 (0.3–28)
Female	14 (50%)
Sweat chloride (mmol/L)	72.50 ± 14.39 (53–109)
Pancreatic insufficiency	21 (75%)
Pseudomonas aeruginosa positive	18 (64%)
BMI (kg/m^2)	19.19 ± 3.27 (11.80–23.75)
BMI-for-age z-score	− 0.45 ± 1.17 (− 3.65–1.00)
ppFEV1	80.00 ± 27.46 (28–133)
FEV1 z-score	− 3.07 ± 1.17 (− 6.68–1.28)
CFTR genotype	*n* (%)
2 Mutations	21 (75)
0 Mutation	4 (14)
Not done	3 (11)
Mutations	*n* (%)
F508del/F508del	8 (29)
F508del/1812-1G > A	2 (7)
F508del/R1066H	2 (7)
F508del/2789 + 5G > A	1 (3.6)
F508del/3132delTG	1 (3.6)
F508del/711 + 1G > T	1 (3.6)
F508del/G542X	1 (3.6)
F508del/p.Arg289AsnfsX17	1 (3.6)
F508del/R1162X	1 (3.6)
F508del/R334W	1 (3.6)
5 T/5 T	1 (3.6)
2307insA/R334W	1 (3.6)
Genotype severity	*n* (%)
Severe	15 (53.6)
Mild	6 (21.4)
Unknown	7 (25.0)
NPD-max (mV)	− 35.14 ± 11.92 (− 76; − 19)
Δ-amil (mV)	24.46 ± 10.38 (9; 51)
Δ-ØCl (mV)	− 0.54 ± 4.47 (− 7; 13)
TCR (mV)	− 1.82 ± 4.15 (− 10; 6)

Data are presented as mean ± standard deviation (range) or frequency (%).

mV millivolts, *BMI* body mass index, *ppFEV$_1$* forced expiratory volume in one second, *NPD-max* maximal nasal potential difference, *Δamil* response to amiloride, *Δ-ØCl* response to a chloride-free solution, containing amiloride, *TCR* total chloride response = sum of response to a chloride-free solution, containing amiloride (Δ-ØCl) + response to chloride-free solution containing amiloride and isoproterenol (Δ-iso)

Table 2 NPD measurements according to pancreatic phenotype, *Pseudomonas aeruginosa* colonization and genotype severity

	NPD-max (mV)	Δ-amil (mV)	ΔØCl	TCR (mV)
Pancreatic phenotype				
PI ($n=21$)	−38.57 (11.40)**	25.71 (10.22)	−0.19 (4.73)	−1.57 (4.34)
PS ($n=7$)	−24.86 (4.19)**	20.71 (9.18)	−1.57 (2.97)	−2.57 (3.02)
Pseudomonas aeruginosa				
Pa+ ($n=18$)	−36.94 (12.61)	25.89 (10.51)	0.56 (4.75)*	−1.78 (3.74)
Pa− ($n=10$)	−31.90 (9.02)	21.90 (9.06)	−2.50 (2.73)*	−1.90 (4.61)
Genotype				
Unknown ($n=7$)	−29.14 (7.14)*	19.71 (9.97)	0.57 (5.68)	−2.43 (4.89)
Mild ($n=6$)	−32.83 (7.41)	28.00 (10.55)	−2.17 (4.14)	−3.67 (3.04)
Severe ($n=15$)	−38.87 (13.92)*	25.27 (10.07)	−0.40 (3.54)	−0.80 (3.80)

Data are presented as mean (± standard deviation)

NPD-max maximal nasal potential difference, *Δ-amil* response to amiloride, *Δ-ØCl* response to a chloride-free solution, containing amiloride, *TCR* total chloride response = sum of response to a chloride-free solution, containing amiloride (Δ-ØCl) + response to chloride-free solution containing amiloride and isoproterenol (Δ-iso), *PI* pancreatic insufficient, *PS* pancreatic sufficient, *Pa Pseudomonas aeruginosa*.

$^{**}P<0.01$, $^{*}P<0.05$

pancreatic phenotype. A significantly more negative NPD-max (−38.57 ± 11.40 mV) ($n=21$) was found in PI patients compared to PS patients (−24.86 ± 4.19) ($n=7$) ($P<0.01$). No association was apparent between the pancreatic status and any other measured parameter (Table 2).

NPD-max was also correlated with genotype severity, being more negative in the severe genotype group (−38.87 ± 13.92 mV), less negative in the mild genotype group (−32.83 ± 7.41 mV) and least negative in the unknown genotype group (−29.14 ± 7.14 mV). The difference was statistically significant between the severe and unknown group ($P=0.04$) (Table 2).

Discussion

Our study showed that the in vivo CFTR-chloride transport in nasal epithelial cells in CF is correlated with better pulmonary function and confirmed that it is also related to genotype, representing a continuous spectrum associated with the genotype severity, appearing lower in patients with two severe CFTR mutations and higher in patients with milder genotype. Our study also showed that CFTR-chloride conductance is strongly associated with pancreatic insufficiency. An interesting finding was the association between Pa with the cellular chloride diffusion through non-CFTR channels.

The cellular basis as well as the precise transport pathways of electrolyte and fluid, absorption and secretion, in the airways are still not well established nor are the primary mechanisms whereby defective CFTR leads to CF airway disease [2, 3, 15–18]. The normal airway epithelium apparently consists of both absorptive and secretory cells whereby fluid and electrolyte transport are balanced across airway epithelia to regulate and maintain homeostasis of the ASL and support efficient MCC [17]. But maintenance of the alkaline ASL-pH based on bicarbonate secretion via CFTR or calcium-activated chloride channel-dependent (CaCCs) is also critical for normal MCC. In CF, the ASL is both more viscous as well as more acidic, impairing the airway MCC and causing mucus retention, airway inflammation, infection, and pulmonary destruction [3–5]. Our results indicate that residual CFTR-chloride conductance is associated with better pulmonary function. Unfortunately, we lack data to correlate CFTR-chloride conductance with structural lung damage, sputum rheology, or MCC measurements. Even though we only assessed the relative CFTR-chloride conductance, it seems possible, if not likely, that the CFTR-bicarbonate transport is affected or spared similarly, which would imply that increased residual CFTR-chloride conductance as well as increased residual CFTR-bicarbonate secretion underlie better clinical outcomes in CF disease.

Pseudomonas aeruginosa is perhaps the most important pathogen in CF lung infection and often associated with more rapid decline in pulmonary function [1, 5]. We found no association between CFTR-chloride conductance with Pa. But, interestingly, we found an association between the magnitude of NPD response to the low-chloride plus amiloride solution-NPD (Δ-ØCl) with Pa infection. The Δ-ØCl represents the chloride diffusion that occurs because of a large chemical gradient generated by a chloride-free solution besides sodium conductance blocking caused by the amiloride. This chloride movement represents mainly the chloride conductance across alternative chloride channels, such as the CaCCs, which also transport bicarbonate [7–9], besides the chloride transport across CFTR. So, it could be possible that maintenance of chloride and bicarbonate transport across alternative channels is involved in bacterial airway clearance, mainly Pa. Of course, our small sample

size limits the significance of this finding, which has to be better studied. Others studies that analyzed the relationship between NPD and Pa infection did not find similar result [19–22], but a study by Green et al. described an association between residual CFTR function, predicted by the class of CFTR mutation, with delayed acquisition of multiple respiratory pathogens in CF, including *Pseudomonas aeruginosa* [23]. In our study, however, no association was found between Pa and genotype severity.

The relationship between NPD and CFTR mutations has been reported for men with congenital bilateral absence of the vas deferens and subjects with non-cystic fibrosis bronchiectasis when used as a diagnostic approach [14, 20]. Wilschanski et al. [14] and Bienvenu et al. [24] reported that the NPD correlates with the number and the severity of the CFTR mutations in atypical CF cases. Our study confirmed that this relationship is also present in a within population of typical CF patients and that shows a pattern of continuity based in genotype severity. The association was demonstrated even though the genotype was unknown in 7 patients, a situation that is not unusual in CF because even extensive mutation analysis may fail to identify mutations in about 4% of patients having a conventional diagnosis of CF [1].

Pancreatic sufficiency is usually associated with better nutritional status and milder pulmonary disease in CF [1], but this association was not seen in our patients. Neither did we find a correlation between PS and residual CFTR-chloride conductance. Similarly, we did not find an association between nutritional condition and CFTR-chloride conductance. The association we found was between PS pancreatic sufficiency and lower cellular hyperpolarization (NPD-max). Initially, the NPD-max was considered as a measure of the upregulated sodium absorption through ENaC that was not inhibited by lack of CFTR function in CF airways [7]. Recent studies in primary cultures of tracheal/bronchial CF epithelia did not confirm that the sodium absorption is increased in CF and the more negative NPD-max was attributed to the loss of anion transport [25]. Thus, the magnitude of the NPD-max seems to be due essentially to the loss of anion conductance, chloride and bicarbonate, and not sodium hyperabsorption. So, based on these results, the defect in CFTR anion conductance is critical in determining pancreatic insufficiency, since bicarbonate is the major anion driving pancreatic duct fluid secretion [26]. Moreover, the pancreas is not known to absorb Na+ or express ENaC [26]. However, previous studies on NPD and CF pancreatic phenotype reported a correlation of residual CFTR function with pancreatic sufficiency [27, 28]. Nonetheless, our results may suffer from a lack of methods that yield a more quantitative evaluation of the pancreatic function in all patients, such as fecal elastase or the fecal fat measurements. Hence, minor malabsorption related to pancreatic insufficiency may not been diagnosed.

The limitations in this study include: (i) The small number of subjects limited solid comparisons between some groups, although sufficient sample size for correlation analysis had been determined. (ii) We used the dermal abrasion NPD-method instead of the subcutaneous reference electrode as suggested by ECFS–NPD–SOP or CFF–TDN–SOP guidelines [9, 10, 13]. We used a dermal abrasion because it was validated in our institution and because of the operator's expertise. De Watcher et al. reported both methods are similar and acceptable suggesting that the operators preferred NPD-method should be used [29]. (iii) The final NPD step with an ATP-solution was not performed. ATP was not available in our laboratory, and we felt that this step is used more as a quality control to confirm the epithelial integrity rather than having essential diagnostic value.

In summary, in vivo less or no anion CFTR-conductance appears to be correlated with CF pulmonary and pancreatic disease. Thus, the CFTR function is the principal factor in CF airway disease pathogenesis and involves anions, chloride and bicarbonate. New therapeutics directed to correct the CFTR- anion conductance and/or the airway acidity are essential in CF, although further studies to elucidate the conductance of chloride and bicarbonate throughout CFTR or alternative channels like CaCCs are needed. Meanwhile, treatments directed to hydrate the airway and improve MCC or correct ASL-pH remain justified.

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Author Contribution EFAP and FAAS design the study. FAAS, PJCM and PMQ provided critical evaluation of methods, results and conclusions. EFAP wrote the manuscript with critique by PJCM and PMQ.

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Compliance with Ethical Standards

Conflict of interest The authors declare no conflicts of interest related to this work.

Informed Consent All subjects or their parents gave and signed a written informed consent. The study was reviewed and approved by the Ethical Review Board of HCPA and registered at HCPA Post-Graduation Research Group as number 05-125.

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