Fatty acid profile in erythrocytes associated with serum cytokines in pediatric cystic fibrosis patients

Perfil de ácidos graxos nos eritrócitos associados com citocinas séricas em pacientes pediátricos com Fibrose Cística

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A B S T R A C T

Objective

To analyze erythrocyte fatty acid composition and its association with serum cytokine levels in pediatric cystic fibrosis patients.

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Methods

A cross-sectional study was performed at a reference center in *Rio de Janeiro*, Brazil. We have included all pediatric patients aged 5-19 years with confirmed cystic fibrosis diagnosis. Erythrocyte fatty acid composition and serum cytokine (TNF-α, IL-1β, IL-6 and IL-8) and C-reactive protein levels were measured. The cut-off point to determine essential fatty acids deficiency was the linoleic acid concentration of <21%.

Results

Twenty-six children (<10 years old) and thirty-one adolescents were studied. Most patients were female and heterozygous for DF508 mutation and suffered from exocrine pancreatic insufficiency. Both children and adolescents had lower linoleic acid concentration (<21%). TNF-α was the only pro-inflammatory marker whose levels were increased; the increase was greater in children. An association between fatty acid composition in erythrocytes and cytokines IL-1β and IL-6 was observed ($p<0.05$).

Conclusion

The pediatric cystic fibrosis patients studied presented a deficiency of essential fatty acids, and an association between fatty acid profile in erythrocytes and serum pro-inflammatory cytokines was observed. These findings highlight the importance of this type of assessment that may open new possibilities for studying pathophysiology and treating cystic fibrosis patients, such as the dietary supplementation with n-3 fatty acids (eicosapentaenoic and docosahexaenoic acids). However, further longitudinal studies are needed for better clarification of the imbalance in lipid metabolism and inflammation in cystic fibrosis

Keywords: Adolescent. Child. Cystic fibrosis. Cytokines. Erythrocytes. Fatty acids.

R E S U M O

Objetivo

Analisar a composição dos ácidos graxos nos eritrócitos e sua associação com os níveis séricos de citocinas em pacientes pediátricos com fibrose cística.

Métodos

Estudo transversal, realizado em um centro de referência no Rio de Janeiro/Brasil. Foram incluídos todos os pacientes pediátricos com diagnóstico confirmado de fibrose cística, entre cinco e 19 anos de idade. Foram analisadas a composição de ácidos graxos nos eritrócitos, as citocinas séricas (TNFα, IL-1, IL-6 e IL-8) e a proteína C reativa. O ponto de corte para determinar a deficiência de ácidos graxos essenciais foi a concentração de ácido linoleico <21%.

Resultados

Foram estudadas 26 crianças (<10 anos) e 31 adolescentes, sendo a maioria do sexo feminino, heterozigoto para a mutação DF508 e com insuficiência pancreática exócrina. Nas crianças e nos adolescentes as concentrações de ácido graxo linoleico eram baixas (<21%). O TNF-α foi o único marcador pró-inflamatório cujas concentrações estavam aumentadas, principalmente nas crianças. Uma associação entre a composição de ácidos graxos nos eritrócitos e as citocinas IL-1β e IL-6 foi observada ($p<0,05$).

Conclusão

Os pacientes pediátricos estudados apresentaram deficiência de ácidos graxos essenciais e foi observada associação entre o perfil de ácidos graxos nos eritrócitos com as citocinas pró-inflamatórias séricas. Os achados destacam a importância deste tipo de avaliação sobre a fisiopatologia e o tratamento de pacientes com fibrose cística, como a suplementação com ácidos graxos n-3 (eicosapentaenoico e docosahexaenoico). No entanto são necessários mais estudos longitudinais no esclarecimento entre o desequilíbrio do metabolismo lipídico e a inflamação na fibrose cística.

Palavras-chave: *Adolescente. Criança. Fibrose cística. Citocinas. Eritrócitos. Ácidos graxos.*

I N T R O D U C T I O N

Airway disease in Cystic Fibrosis (CF) is generally characterized by a continuous cycle of chronic infection and inflammation. Increased production of pro-inflammatory cytokines in the lungs is a key contributor to the pathogenesis of CF lung disease, but what actually initiates this

cycle of inflammation and infection has yet to be elucidated [1].

Chronic inflammation in CF may also contribute to the deficiency in Essential Fatty Acids (EFA). A possible mechanism to explain this association involves increased eicosanoid synthesis due to increased EFA oxidation [2]. At the same time, the imbalance in the metabolism of n-3 and n-6 Polyunsaturated Fatty Acids (PUFA) can result in competition for desaturase and elongase enzymes, which are involved in eicosanoid synthesis, and may promote the formation of pro-inflammatory lipids. Cells involved in the inflammatory response are typically rich in n-6 fatty acids and Arachidonic Acid-derived (AA-derived) eicosanoids, which play an important role in inflammation [3]. Changes in the conversion of Linoleic Acid (LA) to AA trigger an imbalance between AA synthesis and degradation, modulating Cystic Fibrosis Transmembrane Conductance Regulator (CFTR) activity [4,5].

Defects in CFTR activity have been associated with fatty acid disturbances and increased production of pro-inflammatory mediators [2]. Alterations in fatty acid metabolism are therefore considered a primary CF feature rather than a secondary one due to deficient nutrient absorption in these patients. Understanding the mechanisms of fatty acid abnormalities and their association with CF pathophysiology may also be relevant for dietary therapy [6]. In addition, as chronic inflammatory insults are common in CF patients, these individuals have increased levels of inflammatory cytokines and acute phase proteins, including C-Reactive Protein (CRP) [1].

Despite advances in the study of CFTR defects and lung inflammation in CF, the role of fatty acid imbalance in the CF pathophysiology is not fully understood [1,2], and few studies have attempted to correlate the fatty acid profile and inflammatory markers in pediatric CF patients. In the present study, we conducted a cross-sectional study to analyze erythrocyte fatty acid composition and its association with serum pro-inflammatory cytokines in Brazilian pediatric CF patients.

METHODS

This cross-sectional study was conducted at a reference center for pediatric CF treatment in *Rio de Janeiro*, Brazil. All patients aged 5-19 years with confirmed CF diagnosis, treated between January 2009 and December 2010, were included. The medical records were examined, and results of exocrine pancreatic function and pulmonary function were obtained.

Exocrine pancreatic function was assessed by measuring fat or pancreatic elastase-1 content in feces. Patients with more than 5g of fecal fat per day or less than 200mg of elastase-1g of feces were considered to suffer from Exocrine Pancreatic Insufficiency (EPI) [7].

Lung function was assessed based on the Forced Expiratory Volume in the first second (FEV_1). The techniques and reference values followed the recommendations of the American Thoracic Society [8].

Fatty acids in erythrocytes

Blood samples were taken by venipuncture using BD Vacutainer® tubes (Beckton and Dickinson, New Jersey, United States) containing Ethylenediamine Tetraacetic Acid (EDTA) as an anticoagulant after participants had fasted for 12h overnight. Following centrifugation (450g for 7 minutes at 4°C), the fatty acids were extracted from the erythrocytes with n-hexane and isopropanol containing Butylated Hydroxytoluene (BHT) as an antioxidant. The samples were stored at -20°C, evaporated to dryness with nitrogen (N2) and transferred to ultra-freezers (-80°C) until acid methylation [9]. After methylation, N2 was used to evaporate n-hexane from the mixture of Fatty Acid Methyl Esters (FAME), which was then stored at -80°C until chromatographic analysis.

Saturated Fatty Acids (SFA), Monounsaturated Fatty Acids (MUFA), n-6 PUFA, n-3 PUFA and their long-chain derivatives were analyzed by gas chromatography (Agilent Technologies 7890A gas chromatograph) in a standard SP2330 column (60mx320uMx0.2μm) equipped with a split injector and a Flame Ionization Detector (FID). Analysis time was 50 minutes. Initial temperature was 150°C and final temperature 200°C; the carrier gas was hydrogen and the makeup gas was ultrapure nitrogen.

Fatty Acid Methyl Esters (FAME) were identified by comparing their relative retention times with a known standard (GLC-463 reference standard; Nu-Check Prep Inc.) and quantified by comparing the peak area with that of a known internal standard. Results were expressed as mean±Standard Deviation (SD) of weight percentage (g/100g of total fatty acids). The percentage of LA<21% was used as cut-off point to determine EFA deficiency [10].

Inflammatory markers

Concentrations of Tumor Necrosis Factor Alpha (TNF-α), Interleukin-1β (IL-1β), Interleukin-6 (IL-6), Interleukin-8 (IL-8), Prostaglandin E2 (PGE2) and Leukotriene B4 (LTB4) were determined by Enzyme-Linked Immunosorbent Assay (ELISA) using the eBioscience® ELISA kit (Thermo Fisher Scientific, Massachusetts, United States). Plasma samples for ELISA assays were frozen in Eppendorf tubes at -80°C.

Ultrasensitive C-Reactive Protein was measured by nephelometry. Patients were classified as having an inflammation if CRP values were greater than or equal to 0.50mg/dL.

Statistical analysis

Patients were divided into two groups: children (under the age of 10 years) and adolescents (aged 10-19 years). The Kolmogorov-Smirnov test was used to verify the normality assumption. Continuous variables with a normal distribution are expressed as mean and SD, and continuous variables without a normal distribution as median, minimum and maximum. Categorical variables are expressed as absolute and percentage frequencies.

Pearson's chi-square test was used to measure the association among categorical variables, and Fisher's exact test was used when at least one frequency in the chi-square test was lower than five. The *t*-test and Mann-Whitney test were used to compare continuous variables with and without normal distribution, respectively. Pearson and Spearman correlation tests were used to measure the degree of association between two continuous variables with and without normal distribution, respectively. A significance level of $p<0.05$ was adopted. All the statistical analyses were performed using SPSS 20.0 (Statistical Package of the Social Sciences Inc., Chicago, Illinois, United States).

The study was approved by the Research Ethics Committee of the *Instituto Nacional de Saúde da Mulher, da Criança e do Adolescente Fernandes Figueira* (IFF/Fiocruz, National Institute of Women, Children and Adolescents Health Fernandes Figueira) (No 052/07). All the parents of patients gave consent by signing the voluntary informed consent forms.

RESULTS

The study sample consisted of 57 patients (26 children and 31 adolescents) with mean ages of 8±1.4 and 13.5±2.4 years, respectively.

Table 1 shows the clinical characteristics for the two age groups studied and the *p*-value for the association between each characteristic and age. Most patients were female and heterozygous for the DF508 mutation and suffered from EPI. Age of diagnosis was after the second year of life. Pancreatic function and lung function had a significant association with age ($p<0.05$).

Table 1. Clinical characteristics of children and adolescents with Cystic Fibrosis treated at a Cystic Fibrosis reference center in *Rio de Janeiro* (RJ), Brazil, 2009-2010.

Characteristic	Total (n=57)		Children (n=26)		Adolescents (n=31)		*P*-value
	n	%	n	%	n	%	
Age at diagnosis of CF							
<2 years	25	43.9	15	60.0	10	40.0	0.07
≥2 years	32	56.1	11	34.4	21	65.6	
Gender							
Male	24	42.1	11	45.8	13	54.2	1.00
Female	33	57.9	15	45.5	18	54.5	
Genotype							
DF508/DF508	8	14.0	5	62.5	3	37.5	
DF508/other mutation	28	49.1	14	50.0	14	50.0	
Other mutation/other mutation	12	21.1	5	41.7	7	58.3	0.20
Not identified	8	14.0	1	12.5	7	87.5	
Not tested	1	1.8	1	100.0		-	
Pancreatic function							
EPI	46	80.7	24	52.2	22	47.8	0.05*
EPS	11	19.3	2	18.2	9	81.8	
Pulmonary function (%FEV₁ predicted)							
Normal	20	35.1	9	45.0	11	55.0	
Mild obstruction	13	22.8	9	69.2	4	30.7	
Moderate	7	12.3	3	42.9	4	57.1	
Moderate/severe	5	8.8	2	40.0	3	60.0	0.02*
Severe	9	15.8		-	9	100.0	
Very severe	1	1.7	1	100.0		-	
Not measured	2	3.5	2	100.0		-	

Note: *P<0.05.
CF: Cystic Fibrosis; DF508: Delta F508 Mutation; EPI: Exocrine Pancreatic Insufficiency; EPS: Exocrine Pancreatic Sufficiency; FEV1: Forced Expiratory Volume in First Second.

There was no significant difference in erythrocyte fatty acid composition between children and adolescents (Table 2). Both children and adolescents had lower LA concentration (*p*>0.05).

TNF-α was the only inflammatory marker whose levels were different (*p*<0.05) in the two age groups and higher in children than adolescents (Table 3). CRP concentrations were below 0.5mg/dL in most patients.

Table 4 shows the correlation coefficients between fatty acid concentrations and inflammatory cytokines. IL-1β was inversely correlated with palmitoleic acid (C16:1n-7), AA (C20:4n-6) and the palmitoleic acid:AL *ratio*. IL-6 was inversely correlated with the sum of PUFA, stearic acid (C18:0), oleic acid (C18:1n-9) and LA (C18:2n-6) and directly correlated with the AA:AL *ratio*. TNF-α was the only inflammatory marker with a direct correlation with the AA:DHA *ratio*.

DISCUSSION

The LA concentration observed in both children and adolescents in this study was low

Table 2. Erythrocyte-fatty acid composition in children and adolescents with Cystic Fibrosis treated at a cystic fibrosis reference center in *Rio de Janeiro* (RJ), Brazil, 2009-2010.

Fatty acids (%)	Children (n=26)	Adolescents (n=31)	*P*-value
ΣSFA	53.9 ± 10.0	55.5 ± 11.6	0.59
ΣMUFA	16.8 ± 5.4	15.2 ± 5.0	0.25
ΣPUFA	29.3 ± 8.4	29.3 ± 8.4	0.99
ΣPUFA n-6	22.3 ± 6.8	21.8 ± 7.4	0.80
ΣPUFA n-3	7.0 ± 3.0	7.5 ± 3.0	0.52
16:0	27.1 ± 5.5	26.7 ± 5.5	0.78
16:1 n-7	0.2 (0.0 – 1.4)	0.3 (0.0 – 1.7)	0.27
18:0	20.4 ± 4.5	21.3 ± 6.3	0.55
18:1 n-9	8.1 ± 4.3	6.7 ± 3.6	0.19
18:2 n-6	7.0 ± 3.6	6.1 ± 3.5	0.34
18:3 n-3	0.2 ± 0.1	0.3 ± 0.7	0.40
18:3 n-6	1.0 (0.2 – 5.9)	0.9 (0.3 – 8.0)	0.74
20:3 n-6	1.4 ± 1.0	1.5 ± 1.1	0.82
20:4 n-6	6.3 ± 3.4	6.3 ± 3.1	0.96
24:0	3.5 ± 1.7	3.9 ± 1.5	0.31
20:5 n-3	1.0 ± 0.8	1.1 ± 0.6	0.68
22:5 n-6	1.0 ± 1.0	1.0 ± 0.7	0.78
22:5 n-3	1.4 ± 0.8	1.5 ± 0.9	0.46
22:6 n-3	1.2 ± 0.8	1.2 ± 0.9	0.86
n-6:n-3 *ratio*	3.6 ± 1.2	3.3 ± 1.4	0.42
Palmitoleic acid:LA *ratio*	0.0 (0.0 – 2.1)	0.0 (0.0 – 2.6)	0.23
AA:LA *ratio*	1.0 ± 0.4	1.9 ± 2.9	0.11
AA:DHA *ratio*	5.5 ± 2.4	6.9 ± 6.7	0.32

Note: Values expressed as Mean±Standard Deviation or Median (min. – max.).

Σ: Sum; SFA: Saturated Fatty Acids; MUFA: Monounsaturated Fatty Acids; PUFA: Polyunsaturated Fatty Acids; N-6 PUFAs: n-6 Polyunsaturated Fatty Acids; PUFA (n-3): n-3 Polyunsaturated Fatty Acids; 16:0: Palmitic Acid; 16:1n-7: Palmitoleic Acid; 18:0: Stearic Acid; 18:1n-9: Oleic Acid; 18:2n-6: Linoleic Acid (LA); 18:3n-3: Alpha-Linolenic Acid (ALA); 18:3n-6: Gamma-Linolenic Acid (GLA); 20:3n-6: Dihomo-Gamma-Linolenic Acid (DGLA); 20:4 n-6: Arachidonic Acid (AA); 24:0: Lignoceric Acid; 20:5n-3: Eicosapentaenoic Acid (EPA); 22:5n-6: Docosapentaenoic Acid (DPAn-6); 22:5n-3: Docosapentaenoic Acid (DPAn-3); 22:6n-3: Docosahexaenoic Acid (DHA).

(<21%), lower than in pediatric EFA-deficient CF patients reported in other studies (11.23±1.15) [4,11-13]. This result is noteworthy because the concentration of this fatty acid in EPI has become a clinically accepted biomarker for EFA deficiency and it is even more important than the triene:tetraene *ratio* (C20:3n-9:C20:4n-6) [10].

Normal plasma LA status is defined as plasma LA≥21mol% of total fatty acids [10]. Considering that approximately half the children and adolescents had EPI, it is reasonable to suggest that LA reduction may result from β-oxidation of this fatty acid and its conversion into AA to supply energy [2] in order to meet the high metabolic demand in CF patients [14]. Furthermore, most patients in the study had at least one allele with the DF508 mutation. These data may suggest that LA reduction may be associated with CFTR mutations. A correlation between imbalance in fatty acid metabolism and this mutation, in addition to other severe mutations in CF patients, as well as an association between the disorder of EFA metabolism and basic defect of CF, have been described in other studies [6,11,12].

Table 3. Serum inflammatory markers in children and adolescents with Cystic Fibrosis treated at a Cystic Fibrosis reference center in *Rio de Janeiro* (RJ), Brazil, 2009-2010.

Cytokine	Children (n=26)	Adolescents (n=31)	*P*-value
IL-1 β (pg/mL)	12.3 (0.0 – 182.1)	14.1 (0.0 – 168.4)	0.81
IL-6 (pg/mL)	8.3 (1.5 – 42.4)	14.1 (1.1 – 51.4)	0.21
IL-8 (pg/mL)	1.9 (0.0 – 219.7)	3.0 (0.0 – 81.7)	0.59
TNF-α (pg/mL)	36.7 (0.0 – 284.3)	7.2 (0.0 – 337.8)	0.03[*]
PGE$_2$ (pg/mL)	1006.0 (107.6 – 32659.3)	778.2 (73.9 – 5281.5)	0.34
LTB$_4$ (pg/mL)	352.6 (94.2 – 2399.5)	332.0 (96.7 – 2296.9)	0.64
CRP (mg/dL)			
<0.5	18 (47.4)	20 (52.6)	0.78
≥0.5	8 (42.1)	11 (57.9)	

Note: [*]*P*<0.05.

Values expressed as Mean±Standard Deviation and Median (min. – max.) n(%).

IL-1β: Interleukin-1β; IL-6: Interleukin-6; IL-8: Interleukin-8; TNF-A: Tumor Necrosis Factor-A; PGE$_2$: Prostaglandin E$_2$; LTB$_4$: Leukotriene B$_4$; CRP: C-Reactive Protein.

Erythrocyte AA levels in this study were similar to plasma AA levels in CF children (5.99±0.83%) in another study [15]. Nonetheless, the AA levels in CF patients reported in the literature are contradictory since both low [4,11] and elevated values [16,17] have been found. In our study, the high turn-over of n-6 fatty acids and abnormally high AA release in CF, which result in reduced LA, was confirmed [5].

Incorporation of EFA into phospholipids is influenced by chloride channels, suggesting that CFTR may regulate aspects of EFA metabolism. Disturbances in lipid metabolism in CF, such as an increased release of AA from cell membrane phospholipids and low LA and DHA status, have been previously described [4,5]. Different hypotheses have been discussed; however, the link between CFTR, AA release and Docosahexaenoic Acid (DHA) abnormality have yet to be elucidated [2,6].

The concentration of erythrocyte fatty acids better indicates fatty acid status than plasma concentrations when monitoring long-term intake of fatty acids [15,18,19]. DHA, EPA, LA, Alpha-Linoleic Acid (ALA), oleic acid and palmitoleic acid concentrations in erythrocytes were low, which is in agreement with previous studies. The concentration of AA was not as high as that reported in the literature [4,15-17] and an association between erythrocyte fatty acids and IL-1β and IL-6 was observed. These findings indicate that there is an association between PUFA profile and serum pro-inflammatory cytokines in the pediatric CF patients studied.

Analysis of erythrocyte fatty acid composition in children and adolescents in our study showed a higher percentage of SFA (53.9% and 55.5%, respectively) than in an Italian study with CF children aged 6-12 years (35% of total plasma fatty acids) [20].

Although the assessment of nutrient intake was not the goal of the present study, the erythrocyte fatty acid profile suggests that our patients may have a high saturated fat diet. These results are extremely significant because fatty acid concentrations in erythrocytes reflect the intake of fatty acids over the past two months [21]. SFA intake is a risk factor for coronary heart disease [22]. In addition, these patients present chronic inflammation, a known risk factor for atherogenesis [23]. As life expectancy has increased in recent decades for CF patients, high SFA concentrations in erythrocytes can increase the risk of chronic diseases such as coronary heart disease [24].

Table 4. Correlation coefficients between concentrations of fatty acids and inflammatory cytokines in children and adolescents with Cystic Fibrosis treated at a Cystic Fibrosis reference center in *Rio de Janeiro* (RJ), Brazil, 2009-2010.

Fatty Acid	Inflammatory cytokine					
	IL-1 β	IL-6	IL-8	TNF-α	PGE$_2$	LTB$_4$
ΣSFA	-0.08	0.25	0.09	-0.03	0.05	-0.02
ΣMUFA	0.01	-0.07	0.03	-0.01	0.04	0.23
ΣPUFA	0.15	-0.29*	-0.08	0.02	-0.10	-0.09
ΣPUFA n-6	0.19	-0.26	-0.08	0.13	-0.11	-0.08
ΣPUFA n-3	-0.05	-0.18	-0.06	-0.09	-0.06	-0.08
16:0	-0.15	0.13	0.13	-0.05	0.08	0.02
16:1 n-7	-0.27*	-0.14	-0.03	-0.07	-0.03	-0.11
18:0	-0.01	0.28*	0.09	-0.07	0.01	0.00
18:1 n-9	-0.01	-0.28*	0.03	-0.05	-0.12	0.14
18:2 n-6	0.05	-0.28*	0.06	-0.05	-0.16	0.07
18:3 n-3	-0.07	0.01	0.00	-0.19	-0.18	0.14
18:3 n-6	-0.05	-0.10	0.13	0.06	0.01	0.04
20:3 n-6	0.15	-0.19	-0.03	0.02	-0.18	-0.00
20:4 n-6	0.27*	-0.23	-0.11	0.11	-0.03	-0.16
24:0	-0.07	0.22	-0.11	-0.14	0.03	-0.15
20:5 n-3	0.05	-0.05	0.05	0.14	0.22	-0.09
22:5 n-6	0.05	-0.08	0.11	0.12	0.15	-0.08
22:5 n-3	0.10	0.00	-0.05	-0.00	-0.15	-0.11
22:6 n-3	0.19	-0.25	-0.26	-0.03	-0.12	-0.13
n-6:n-3 *ratio*	0.14	0.06	0.08	0.13	-0.05	0.12
Palmitoleic acid:LNA *ratio*	-0.27*	0.18	-0.06	-0.06	-0.01	-0.14
ARA:LNA *ratio*	0.23	0.37*	-0.17	0.23	0.05	-0.14
ARA:DHA *ratio*	0.07	0.14	0.19	0.31*	0.01	0.05

Note: *$P<0.05$

IL-1B: Interleukin-1β; IL-6: Interleukin-6; IL-8: Interleukin-8; TNF-A: Tumor Necrosis Factor A; PGE$_2$: Prostaglandin E$_2$; LTB$_4$: Leukotriene B$_4$; CRP: C-Reactive Protein; Σ: Sum; SFA: Saturated Fatty Acids; MUFA: Monounsaturated Fatty Acids; PUFA (n-6): n-6 Polyunsaturated Fatty Acids; PUFA (n-3): n-3 Polyunsaturated Fatty Acids; C16:0: Palmitic Acid; C16:1 n-7: Palmitoleic Acid; C18:0: Stearic Acid; C18:1n-9: Oleic Acid; C18:2n-6: Linoleic Acid (LNA); C18:3n-3: Alpha-Linolenic Acid (ALA); C18:3n-6: Gamma-Linoleic Acid (GLA); C20:3n-6: Dihomo-Gamma-Linolenic Acid (DGLA); C20:4n-6: Arachidonic Acid (AA); C24:0: Lignoceric Acid; C20:5n-3: Eicosapentaenoic Acid (EPA); C22:5n-6: Docosapentaenoic Acid (Dpan-6); C22:5n-3: Docosapentaenoic Acid (Dpan-3); C22:6n-3: Docosahexaenoic Acid (DHA); LNA: Linoleic Acid; ALA: Alpha-Linolenic Acid; ARA: Arachidonic Acid; EPA: Icosapentaenoic Acid; DHA: Docosahexaenoic Acid.

The levels of DHA and EPA in erythrocytes in our study were similar to those observed in plasma and tissues of EFA-deficient CF children in a previous study (1.23±0.18 and 0.94±0.09, respectively) [15]. Other authors have also observed low plasma and tissue DHA concentrations [4,11-13,16,25,26] as well as low erythrocyte EPA levels in CF patients [19,27]. DHA and EPA are converted to resolvins and protectins, molecules that can act as anti-inflammatory and inflammation-resolving mediators [3]. Erythrocyte DHA and EPA levels are considered long-term markers for bioavailability and delivery of Long-Chain n-3 Polyunsaturated Fatty Acids (LC-PUFA) [28]. Nevertheless, there is substantial evidence that less than 1% of dietary ALA is converted into DHA through the n-3 pathway [29]. These data may suggest the

importance of therapeutic supplementation with LC n-3 PUFA, such as EPA and DHA, for improving dietary therapy in CF patients.

Although DHA and EPA have the most potent anti-inflammatory effects, the therapeutic dose of n-3 PUFA in CF, a multifactorial disease, will also depend on the degree of severity of the disease, which, in turn, depends on the patient's genetic predisposition and individual factors that affect the bioavailability of n-3 PUFA [2,28]. Several studies have shown that the optimal n-6:n-3 ratio may vary among diseases. Furthermore, there is no consensus on the ideal LA:ALA ratio intake in CF because of the imbalance and deficiency of LA and DHA in these patients [2,4]. Evidences are insufficient to understand the relationship between diet and inflammation and the ideal n-6:n-3 ratio for CF [6].

Our results confirmed that there was a low concentration of ALA, as it was observed in other studies with CF patients that found the following levels of erythrocytes and plasma fatty acids, 0.43±0.71% and 0.2%, respectively [20,25].

We found a higher AA:LA ratio than those observed in pediatric CF patients in Spain (0.27 and 0.28) [26], possibly indicating that the pathway from LA to AA was enhanced in children and adolescents in our study. In CF, the conversion of AL to AA is unregulated due to increased activity of the enzyme Phospholipase A2 (PLA2), which promotes an increase in AA, reducing the concentration of AL and DHA [2,5]. Our results support this hypothesis.

The AA:DHA ratio in our study was higher than the one found in individuals without CF, corroborating several studies [17,20,26]. When this ratio is high in CF patients, it is believed to be an important indicator of the inflammatory state [12], reflecting the ability of AA to generate eicosanoids that promote the production of pro-inflammatory cytokines and may be responsible for the early development of pulmonary disease [2,3]. This may explain the

direct correlations found between TNF-α and the AA:DHA ratio, IL-6 and the AA:LA ratio, and IL-1 and AA observed in this study.

The concentrations of palmitoleic and oleic acids (MUFA profile) in erythrocytes were lower in both groups than those found in another study, 0.9±0.4% [30] and 17.9±4.4 [25], respectively. The lower concentrations of oleic acid may be a result of conversion of this acid into eicosatrienoic acid, which was not measured in the present study. Although they are not eicosanoid precursors, MUFA may influence synthesis of these derivatives and the lipid metabolism of PUFA through enzymatic inhibition and saturation [18].

In EFA deficiency, the level of desaturases increases, despite the lower affinity of oleic acid for delta-6-desaturase, and this acid is converted into large quantities of eicosatrienoic acid [15]. The erythrocyte palmitoleic acid:LA ratio was lower in adolescents than in children. This ratio is also considered an EFA deficiency marker in plasma, erythrocyte membranes and platelets in CF and it is a better marker than the triene:tetraene ratio [15]. This could explain the indirect correlation between IL1-β and the palmitoleic acid:LA ratio observed in our patients.

There are no general cut-off or reference values for plasma concentrations of the inflammatory markers studied in our study, as cytokines are synthesized by a variety of cell types, and plasma measurements may not reflect tissue inflammation in CF-related organs [31]; for example, inflammation caused by a specific pathogen is normally compartmentalized in the lung. Similarly, erythrocyte membrane fatty acid levels do not always reflect any increase or decrease in these levels at the inflammation site [32]. Thus, circulating cytokines may not be the best way to assess the intensity of inflammation in CF as their bioavailability may be modulated by soluble receptors and natural inhibitors [32]. In addition, cytokine levels were measured at a single time point and hence cannot be

considered to reflect the level of inflammation to which CF patients are exposed during their disease. Moreover, most of our patients showed no evidence of acute inflammation, as revealed by their CRP levels (<0.5mg/dL). These are elevated when CF is exacerbated, and they can reveal early inflammation when other clinical parameters are equivocal [33].

TNF-α can be considered an early marker of inflammation in pediatric patients. Surprisingly, levels of this marker were extremely high in the children in our study and it was the only pro-inflammatory marker whose levels were statistically different in the two age groups. This finding is important because elevated plasma levels of TNF-α are also associated with reduced lean body mass, increased muscle proteolysis, increased respiratory exacerbations and poorer lung function, even in clinically stable patients [34].

As there are few studies comparing the profile of fatty acids and inflammatory markers in pediatric patients with CF, the scope for interpretation and comparison of our findings with the literature was limited. This was the first study conducted in Brazil to evaluate the composition of erythrocyte fatty acids and correlate them with inflammatory markers in pediatric CF patients.

CONCLUSION

The pediatric CF patients presented EFA deficiency. An association between fatty acid profile in erythrocytes and serum pro-inflammatory cytokines was observed in the present study and imbalance in lipid metabolism may be associated with CFTR mutations.

These findings highlight the importance of assessing erythrocyte fatty acids and serum pro-inflammatory cytokines that may open new possibilities for studying the pathophysiology and treating CF patients, such dietary supplementation with n-3 fatty acids (EPA and DHA). However, further longitudinal studies are needed for better clarification of the imbalance in lipid metabolism and inflammation in patients with CF.

CONTRIBUTORS

ALP Cunha conceptualized and designed the study, coordinated and supervised data collection, drafted the initial manuscript and made final revisions based on the critical reviews of the other authors. AC COSTA coordinated and carried out the statistical analyses, reviewed the manuscript and approved the final version of the manuscript. Z VASCONCELOS coordinated and carried out the cytokine measurements, reviewed the manuscript and approved the final version of the manuscript. MG TAVARES DO CARMO supervised the study, coordinated the fatty acid measurements, critically reviewed the manuscript and approved the final version of the manuscript. CR CHAVES conceptualized and designed the study, supervised the study, drafted the initial manuscript, critically reviewed the manuscript and approved the final version of the manuscript.

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