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Original Article

TNF-alpha polymorphisms as a potential modifier gene in the cystic fibrosis

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Abstract: Modifier genes, as the *TNF-α* gene, can modulate the cystic fibrosis (CF) severity. Thus, -238G>A and -308G>A polymorphisms of *TNF-α* gene were analyzed as modifiers of CF. In this context, the present study enrolled 49 CF patients (diagnosis performed by sweat test and complete *CFTR* screening). The -238G>A polymorphism analysis was performed by ARMS-PCR, and -308G>A, by PCR-RFLP. In our data, the -238G>A polymorphism was not associated with clinical variability. The AA genotype for -308G>A polymorphism was a risk factor for early gastrointestinal symptoms (OR=5.98, 95%CI=1.06-49.68) and protection for the first *Pseudomonas aeruginosa* (OR=0.05, 95%CI=0.0003-0.007). For the first *P. aeruginosa*, GA genotype was a risk factor (OR=10.2, 95%CI=1.86-84.09); for the same genotype, the diagnosis was made in minor time than the AA genotype (p=0.031). Considering the -308G>A polymorphism alleles, the G allele was a risk factor for early pulmonary symptoms (OR=3.81, 95%CI=1.13-12.97) and *P. aeruginosa* (OR=66.77, 95%CI=15.18-482.7); however, the same allele showed better transcutaneous oxygen saturation (OR=9.24, 95%CI=1.53-206.1). The A allele was a protective factor for early pulmonary symptoms (OR=12.26, 95%CI=0.08-0.89) and *P. aeruginosa* (OR=12.15, 95%CI=0002-0007), however, the same allele was a risk factor for worst transcutaneous oxygen saturation (OR=7.01, 95%CI=1.14-157.4). As conclusion, the -308G>A polymorphism of the *TNF-α* gene was associated with the CF severity.

Keywords: Cystic fibrosis, genotype, phenotype, polymorphism, *TNF-α*, TNF-alpha, *CFTR*, lung disease, inflammation, modifier gene

Introduction

Cystic fibrosis (CF) has high clinical variability conditioned by environmental factors, *CFTR* and modifier genes [1-3]. Our group has studied the association of modifier genes with clinical markers of CF, among the studied genes, we can highlight: *MBL-2*, *TGF-β1*, *CD14* [4], *GSTM1*, *GSTT1* [5, 6], *ACE* [7], *ADRB2* [8], *TCF7L2* [9], *COX-2* [10], *ADRA2A* [11], *IFRD1* [12], *GSTP1* and *GCLC* [6].

Among the genes with possible modifier effect of the CF severity, the *TNF-α* (region 6p21.3) has highlight. *TNF-α* gene is associated with the expression of tumor necrosis factor alpha by polymorphism in the promoter region, acting on transcriptional and post-transcriptional levels [13, 14]. Some polymorphisms were associated with the severity of lung disease in CF [15] and infection by *Pseudomonas aeruginosa* [16]. The tumor necrosis factor has been found with high concentrations in the lungs of CF patients, suggesting action in the inflammatory lung disease [15, 17].

The most studied polymorphisms are characterized by the exchange of a guanine by an adenine at positions -238 (rs361525) and -308 (rs1800629). The -238A>G polymorphism shows conflicting results acting in TNF expression [18, 19], including in the CF [20]. While the -308A>G polymorphism was associated with the defense response produced by *TNF-α* [21]. The G allele (-308A>G polymorphism) is the rare allele and was associated with increased gene transcription and inflammatory process [21, 22], being in CF, the allele responsible for

the worst clinical presentation [15], lowest pulmonary function and lower body mass index (BMI) [23, 24].

TNF-α protein is a multifunctional pro-inflammatory cytokine produced primarily by macrophages, being the main mediator of the acute inflammatory response to microorganisms, as well, responsible for systemic complications [25]. The main function of TNF-α is stimulate the recruitment of neutrophils and monocytes to sites of infection and activate these cells to eradicate microorganisms. TNF-α induces the vascular endothelial cells to express adhesion molecules that become the endothelial surface adhesive for leukocytes in the site of infection [25, 26]. The TNF-α protein has function is the regulation of biological processes, such as: (i) cellular proliferation and differentiation, (ii) apoptosis, (iii) metabolism of lipids, and (iv) blood coagulation. Considering the importance of TNF protein and modulation of its expression by polymorphisms in *TNF-α* gene, the -238G>A and -308G>A polymorphisms in the regulatory region were analyzed and compared with clinical variables of CF patients with CF.

Material and methods

Casuistic

49 CF patients diagnosed by the sweat test were enrolled (sodium and chloride with values above 60 mEq/L). All patients were homozygous or compound heterozygous for mutations Class I or II in the *CFTR* gene. No patient had diagnosis performed by neonatal screening test. The study was approved by the Ethics Committee from our university (#570/2004).

DNA extraction

Patients' DNA was obtained by phenol-chloroform extraction. The DNA concentration used for analysis was 50 ng/mL, evaluated using GE NanoVue™ Spectrophotometer (GE Healthcare Biosciences, Pittsburgh, USA).

TNF-α polymorphisms screening

The polymerase chain reaction (PCR) reaction for amplification of the *TNF-α* polymorphisms was performed with bidistilled water, 10x Taq buffer with $(NH_4)_2SO_4$, $MgCl_2$ (25 mM), dNTP (25 mM each nitrogenous base), primers (0.2 pmol), Taq polymerase (5U) and genomic DNA (50 ng/mL). For the -238A>G polymorphism was realized an amplification-refractory mutation system (ARMS)-PCR, being respectively for the internal control [5'-GCCCCTCCAGTTCTAGTTCTATC-3' and 5'-CCGGATCATGCTTTCAGTGC-3'], allele A [5'-GCCCCTCCCAGTTCTAGTTCTATC-3' and 5'-CACACTCCCATCCTCCCTGGTCT-3'] and allele G [5'-AGACCCCCCTCGGAATCG-3' and 5'-CCGGATCATGCTTTCAGTGC-3'], observed a fragment of 608, 209 and 447 basis pair. For the -308A>G [primers sense 5'-AGGCAATAGGTTTTGAGGGCCAT-3' and antisense 5'-GAGCGTCTGCTGGCTGGGTG-3'] polymorphism a fragment of 345 basis pair was amplified, follow by restriction fragment length polymorphism (RFLP) technique.

The PCR digestion was performed with the enzyme *NcoI* (New England BioLabs, Ipswich, MA). Before the digestion, the purification was performed with 10 μL of the PCR and 80 μL of isopropyl alcohol (75%), follow by 10 min on 25°C, and after centrifuged at 13,000 rpm for 30 min. The tube was inverted and was added 150 μL of ethyl alcohol (70%) at room temperature and again centrifuged at the same speed for 10 min. After centrifugation, the tube was inverted and allowed to dry at room temperature, and 10 μL of water was added. From this volume, was added 2.0 uL of enzyme specific buffer (10 mM Tris-HCl pH 7.5; 50 mM KCl; 0.1 mM EDTA; 1 mM DTT; 0.2 mg/mL BSA and 50% glycerol), 7.5 mL of water and 0.5 mL of enzyme (*NcoI*) (5U). Digestion was carried out at 37°C. The G allele is not digested. The A allele represents the restriction site (GG - 345 basis pair, GA - 345, 320 and 25 basis pair, AA - 320 and 25 basis pair).

Clinical variables

The clinical data included in the study were: *CFTR* mutations, age at diagnosis, age at onset of pulmonary and digestive symptoms, first isolation by *P. aeruginosa*, spirometry, Shwachman-Kulczycki and Kanga scores, and transcutaneous oxygen saturation of hemoglobin.

For age at diagnosis, age at onset of pulmonary and digestive symptoms, and time for the first isolation of *P. aeruginosa*, the following groups were included: under 12 months, from 13 to 36 months, above 36 months. In the case of digestive symptoms was considered meconium ileus as an additional risk factor. For *P. aeruginosa*,

Table 1. Population characterization for the *TNF-α* and *CFTR* genotypes, Shwachman-Kulczycki and Kanga scores, transcutaneous oxygen saturation of hemoglobin and forced expiratory volume in the first second (%) (FEV_1) of forced vital capacity

Patient	*TNF-α* genotype		*CFTR* genotype	Shwachman-Kulczycki	Kanga	Oxygen saturation	FEV_1%
	-238G>A	-308G>A					
MVG	G/G	G/A	F508del/F508del	Moderate	Normal	Normal	Normal
JPD	G/G	G/A	F508del/F508del	****	Normal	Normal	Mild
RSR	G/A	A/A	F508del/F508del	Excellent	Normal	Mild	Normal
EVM	G/G	A/A	F508del/R1162X	Excellent	Normal	Mild	Severe
IFM	G/G	A/A	F508del/F508del	****	****	****	****
MLA	G/G	A/A	F508del/F508del	****	Exacerbate	Normal	Moderate
VLPC	G/G	A/A	F508del/F508del	Moderate	Normal	Normal	Normal
MAB	G/G	A/A	F508del/F508del	Severe	Exacerbate	Mild	Severe
EVSMS	G/G	A/A	F508del/F508del	****	Normal	Normal	Mild
YBK	G/G	A/A	F508del/F508del	Moderate	Normal	Normal	Normal
HB	G/A	A/A	F508del/G542X	****	Normal	Severe	Severe
WSP	G/G	A/A	F508del/G542X	Moderate	Normal	Mild	Severe
DRG	G/G	A/A	F508del/F508del	****	****	****	****
BBK	G/G	G/A	F508del/N1303K	****	****	****	****
AO	G/G	A/A	F508del/F508del	****	Exacerbate	Mild	Mild
LSM	G/A	A/A	F508del/F508del	Excellent	Normal	Normal	Moderate
VAL	G/G	A/A	F508del/F508del	Good	Normal	Mild	Mild
LFSA	G/G	A/A	F508del/F508del	****	Normal	Mild	Mild
IBN	G/G	A/A	F508del/R553X	Excellent	Normal	Normal	Normal
CAQ	G/G	G/G	F508del/G542X	****	Normal	Normal	Mild
MEMZ	G/A	A/A	F508del/F508del	****	Normal	Normal	Normal
TMG	G/G	G/G	F508del/F508del	Moderate	Normal	Normal	Normal
JMGR	G/G	G/A	F508del/N1303K	****	Normal	Normal	****
MAP	G/A	G/G	F508del/F508del	****	****	****	****
AX	G/G	A/A	F508del/F508del	Good	Normal	Normal	Normal
LPOL	G/G	G/A	F508del/N1303K	Good	Normal	Normal	****
EG	G/G	A/A	F508del/R1162X	Good	Normal	Normal	Normal
NCB	G/G	G/A	F508del/R553X	****	Normal	Mild	Severe
FVV	G/G	A/A	F508del/G542X	Excellent	Normal	Normal	Normal
LSS	G/G	A/A	F508del/F508del	****	Exacerbate	Moderate	****
RNC	G/G	A/A	F508del/F508del	****	Normal	Normal	****
FEL	G/G	A/A	F508del/G542X	Moderate	Normal	Normal	Moderate
GOV	G/G	G/A	F508del/F508del	****	Normal	Normal	****
CVAR	G/G	A/A	F508del/G542X	****	Normal	Normal	****
EAG	G/G	A/A	F508del/F508del	****	Normal	Mild	****
AVM	G/G	G/G	DF508/G542X	Excellent	Normal	Normal	Normal
BSG	G/G	A/A	G542X/R1162X	Good	Exacerbate	Mild	Mild
GPNS	G/A	A/A	F508del/F508del	****	Normal	Normal	****
CAL	G/G	A/A	DF508/G542X	Good	Normal	Normal	Normal
MOS	G/G	G/G	F508del/F508del	Moderate	Normal	Normal	****
FSC	G/G	A/A	F508del/F508del	Good	Normal	Normal	Normal
ITVS	G/G	G/A	F508del/F508del	****	Normal	Normal	Normal
JRL	G/G	G/G	F508del/F508del	Mild	Normal	Normal	****
GPT	G/A	A/A	F508del/F508del	Excellent	Normal	Normal	Normal

ASR	G/A	G/A	F508del/R1162X	****	****	****	****
VAF	G/G	A/A	F508del/F508del	****	Normal	Normal	****
IRO	G/A	G/G	F508del/F508del	****	Normal	Normal	****
BMSL	G/G	A/A	R1162X/R1162X	****	****	****	****
BSC	G/A	A/A	F508del/F508del	Moderate	Normal	Normal	Mild

****: no data; *CFTR* = Cystic Fibrosis Transmembrane Regulator; *TNF-α* = tumor necrosis factor.

Table 2. Patients distribution according with the clinical characteristics taking into account categorical data

Clinical variable	Category	Number of patients (%)
Age at diagnosis	<12 months	27 (56.25)
	13 to 36 months	8 (16.66)
	>36 months	13 (27.08)
Onset of pulmonary symptoms	<12 months	33 (70.21)
	13 to 36 months	7 (14.89)
	>36 months	5 (10.64)
	No clinical symptom	2 (4.25)
Onset of digestive symptoms	<12 months	32 (68.08)
	13 to 36 months	4 (8.51)
	>36 months	4 (8.51)
	Meconium ileus	7 (14.89)
	No clinical symptoms	1 (2.12)
Age for the first infection by *Pseudomonas aeruginosa*	<12 months	13 (27.66)
	13 to 36 months	14 (29.79)
	>36 months	15 (31.91)
	Without bacteria	5 (10.64)
Kanga score	Non exacerbation	38 (88.37)
	Exacerbation	5 (11.36)
Shwachman-Kulczycki score	Excellent	7 (29.16)
	Good	7 (29.16)
	Moderate	8 (33.33)
	Mild	1 (4.16)
	Severe	1 (4.16)
Transcutaneous oxygen saturation of hemoglobin	Normal	32 (72.72)
	Mild	10 (22.72)
	Moderate	1 (2.27)
	Severe	1 (2.27)
Forced expiratory volume in first second of the FVC	Normal	15 (51.72)
	Mild	8 (27.59)
	Moderate	3 (10.34)
	Severe	3 (10.34)

FVC = forced vital capacity.

some patients were decolonized until the beginning of the study period, being not included in the statistical analysis.

In the spirometry test was considered severe obstruction, values for the forced expiratory volume in one second (FEV_1%) minor than 40% of the predicted, moderate obstruction, values between 40 to 60%, mild obstruction, values between 60 to 80%, and normal lung function, values greater than 80%. In data analysis, the FEV_1% mean was used for the classification of

Table 3. Genotypes for the -238G>A and -308G>A polymorphisms of the *TNF-α* gene in cystic fibrosis patients

Polymorphism	Genotype	Number of patients (%)
-238G>A*	G/G	39 (79.59)
	A/A	0
	G/A	10 (20.41)
	Total	49
-308G>A#	GG	7 (14.28)
	GA	9 (18.36)
	AA	33 (67.35)
	Total	49

TNF-α = tumor necrosis factor. *Hardy-Weinberg equilibrium - p>0.05; χ^2=2.0. Allelic frequency: G allele = 0.90, A allele = 0.10. #Hardy-Weinberg equilibrium - p>0.05; χ^2=2.57. Allelic frequency: G allele = 0.23, A allele = 0.77.

severity, being compared minor and greater values than the median.

The Spirometry proof was performed using a speedometer model CPFS/D (Med Graphics, Saint Paul, Minnesota, USA). Data were recorded by the BREEZE PF Version 3.8 B software for Windows 95/98/NT.

The Shwachman-Kulczycki score is graded as excellent (86-100), good (71-85), mild (56-70), moderate (41-55) and severe (40 or less), taking into account the number of points. In the study, the categories were compared between the polymorphisms analyzed directly, considering the possible genotypes and alleles, and reassembled, as follow: excellent and good scores became mild, the mild score became moderate, and moderate and severe scores were defined as severe. The distribution of scores in three categories was performed to allow statistical analysis considering the number of patients included in the study. The score was evaluated by two professionals of the Pediatric Clinic of the Clinical Hospital of the Faculty of Medical Sciences, to better evaluation, being a subjective analysis. In case of disparity between evaluators, third evaluation was performed by a qualified professional [27].

The score Kanga was analyzed considering the presence or absence of exacerbation through the points obtained in the analysis [27].

Transcutaneous oxygen saturation of hemoglobin was used in the classification: saturation greater than 95% - normal value, between 91 to 95 - mild hypoxemia, between 85 to 90 - moderate hypoxemia and minor than 8% - severe hypoxemia.

Statistical analysis

The statistical analysis was performed by Statistical Package for Social Sciences version 21.0 (SPSSv.21). The Kruskal-Wallis test and T-student test were conducted to assess the interaction between the phenotypic criteria with numerical distribution, and the χ^2 test or Fisher's exact test for categorical variables, with polymorphisms of the *TNF-α* gene, taking into account the genotypes and alleles. In the data analysis was considered α=0.05.

Results

Of the 49 subjects enrolled, the age ranged from one to 26 years, with a mean of 9.71 years (± 6.06 years). Distribution by age group showed 34 patients between zero and 10 years, 12 between 11 and 21, and three above 21 years. 25 women (51.02%) was included. The mean age of diagnosis was 29.70 months (± 2.47 months). For the first isolation of *P. aeruginosa*, the mean age was 38.11 months (± 3.17 months).

Considering *CFTR* genotype, F508del homozygous predominated (63.26%). The F508del allele had the highest prevalence (79.59%), followed by G542X (9.18%), R1162X (6.12%), N1303K (3.06%) and R553X (2.04%).

The results for *TNF-α* and *CFTR* genotype, Shwachman-Kulczycki and Kanga scores, transcutaneous oxygen saturation of hemoglobin and FEV_1% are shown in **Table 1**. The population description for the clinical variables with categorical distribution is shown in **Table 2**. In **Table 3**, the frequency of genotypes for the polymorphisms of *TNF-α* gene is described, including the allele frequency and Hardy-Weinberg equilibrium. In the population studied, both polymorphisms are in balance.

In **Tables 4**-**6** are described the associations of the -238G>A polymorphisms of the *TNF-α* gene, considering, respectively, clinical categorical variables and genotypes for the polymorphism, clinical categorical variables and allele frequency for the polymorphism, and numeric clinical variables and genotypes/allele for the polymorphism. For the polymorphism -238G>A, no association with clinical variables was observed.

Table 4. Association of -238G>A polymorphism (genotypes) of the *TNF-α* gene with clinical variables of cystic fibrosis patients

Clinical variables	Groups	Genotypic distribution			
		G/G	OR (95%CI)	G/A	OR (95%CI)
Age at diagnosis	<12 months	4	0.44 (0.09-1.89)	23	2.26 (0.53-10.49)
	13 to 36 months	4	5.39 (0.98-30.7)	4	0.19 (0.03-1.02)
	>36 months	2	0.62 (0.08-3.22)	11	1.61 (0.31-12.67)
Onset of pulmonary symptoms	<12 months	6	0.53 (0.12-2.56)	26	1.90 (0.39-8.64)
	13 to 36 months	2	1.49 (0.17-9.18)	5	0.67 (0.11-5.81)
	>36 months	2	1.91 (0.21-12.84)	4	0.52 (0.08-4.72)
	No clinical symptom	0	-	2	-
Onset of digestive symptoms	<12 months	7	0.88 (0.15-7.54)	24	1.14 (0.13-6.89)
	13 to 36 months	2	3.82 (0.35-42.28)	2	0.26 (0.02-2.88)
	>36 months	0	-	4	-
	No clinical symptom	0	-	1	-
Age for the first infection by *Pseudomonas aeruginosa*	<12 months	4	1.93 (0.39-9.10)	11	0.52 (0.11-2.54)
	13 to 36 months	3	1.22 (0.22-5.87)	11	0.82 (0.17-4.63)
	>36 months	2	0.71 (0.09-3.79)	11	1.42 (0.26-11.3)
	No clinical symptom	0	-	5	-
Forced expiratory volume in first second of the FVC	Normal (>80%)	3	1 (0.14-6.90)	12	1 (0.14-6.90)
	Mild (60-80%)	1	0.61 (0.02-5.66)	6	1.64 (0.18-45.91)
	Moderate (40-60%)	1	2.13 (0.06-33.11)	2	0.47 (0.03-15.98)
	Severe (<40%)	1	1 (0.03-10.34)	4	1 (0.10-29.45)
Shwachman-Kulczycki score	Excellent (86-100)	4	2.35 (0.37-16.63)	3	1.36 (0.19-8.90)
	Good (71-85)	1	0.16 (0.006-1.39)	6	6.26 (0.72-172.2)
	Mild (56-70)	0	-	1	-
	Moderate (41-55)	4	1.63 (0.27-10.02)	4	0.61 (0.10-3.69)
	Severe (≤40)	1	-	0	-
Kanga score	Exacerbated	2	2.86 (0.29-22.87)	3	0.35 (0.04-3.41)
	Non exacerbated	7	0.35 (0.04-3.41)	31	2.86
Transcutaneous oxygen saturation of hemoglobin	Normal (>95%)	9	4.38 (0.60-107.9)	22	0.23 (0.009-1.66)
	Mild (91-95%)	1	0.30 (0.01-2.26)	9	3.30 (0.44-82.39)
	Moderate (85-90%)	0	-	1	-
	Severe (<85%)	0	-	1	-

TNF-α = tumor necrosis factor; FVC = forced ventilator capacity. Statistical analysis performed by χ^2 test and Fisher's exact test.

In **Tables 6-8** are described associations of the -308G>A polymorphisms of the *TNF-α* gene, considering, respectively, numeric clinical variables and genotypes/allele for the polymorphism, clinical categorical variables and genotypes for the polymorphism, and clinical categorical variables and allele frequency for the polymorphism.

The AA genotype for -308A>G polymorphism was a risk factor for early onset of digestive symptoms (under 12 months) (OR=5.98, 95%CI=1.06-49.68) and protection for the first isolation of *P. aeruginosa* (under 12 months) (OR=0.05, 95%CI=0.0003-0.007). For the first *P. aeruginosa*, the heterozygous genotype was a risk factor for early isolation (under 12 months) (OR=10.2, 95%CI=1.86-84.09); and for the same genotype, the diagnosis was made in minor time than the AA genotype (p=0.031).

Considering the alleles for the -308G>A polymorphism, the G allele was a risk factor for early onset of pulmonary symptoms (OR=3.81, 95%CI=1.13-12.97), and for the first *P. aeruginosa* (under 12 months) (OR=66.77, 95%CI=15.18-482.7), however, the same allele was a protective factor for better values of transcutaneous oxygen saturation (>95%) (OR=9.24, 95%CI=1.53-206.1). The A allele

Table 5. Association of -238G>A polymorphism (alleles) of the *TNF-α* gene with clinical variables of cystic fibrosis patients

Clinical variables	Groups	Allelic distribution			
		G allele	OR (95%CI)	A allele	OR (95%CI)
Age at diagnosis	<12 months	50	2.07 (0.53-8.86)	4	0.48 (0.11-1.89)
	13 to 36 months	12	0.25 (0.06-1.13)	4	4.03 (0.89-17.01)
	>36 months	24	1.54 (0.33-11.29)	2	0.65 (0.09-3.05)
Onset of pulmonary symptoms	<12 months	58	17.46 (0.40-6.99)	6	0.57 (0.14-2.50)
	13 to 36 months	12	0.71 (0.14-5.40)	2	1.41 (0.19-7.05)
	>36 months	10	0.58 (0.11-4.46)	2	1.74 (0.22-8.91)
	No clinical symptom	4	-	0	-
Onset of digestive symptoms	<12 months	55	1.12 (0.15-5.71)	7	0.89 (0.18-6.86)
	13 to 36 months	6	0.34 (0.06-2.85)	2	2.94 (0.35-17.31)
	>36 months	8	-	0	-
	No clinical symptom	2	-	0	-
Age for the first infection by *Pseudomonas aeruginosa*	<12 months	26	0.67 (0.16-3.01)	4	1.5 (0.33-6.42)
	13 to 36 months	25	1 (0.23-5.25)	3	1 (0.19-4.37)
	>36 months	24	1.64 (0.34-12.25)	2	0.61(0.08-2.98)
	No clinical symptom	10	-	0	-
Forced expiratory volume in first second of the FVC	Normal (>80%)	27	1 (0.16-6.29)	3	1 (0.16-6.29)
	Mild (60-80%)	13	1.57 (0.19-40.47)	1	0.64 (0.02-5.13)
	Moderate (40-60%)	5	0.52 (0.06-14.46)	1	1.93 (0.07-18.17)
	Severe (<40%)	9	1 (0.12-26.31)	1	1 (0.04-8.40)
Shwachman-Kulczycki score	Excellent (86-100)	10	0.54 (0.12-2.59)	4	1.84 (0.39-8.22)
	Good (71-85)	13	4.56 (0.64-110.9)	1	0.22 (0.009-1.56)
	Mild (56-70)	2	-	0	-
	Moderate (41-55)	12	0.70 (0.16-3.28)	4	1.43 (0.31-6.26)
	Severe (≤40)	1	0.25 (0.006-10.51)	1	3.95 (0.10-164.2)
Kanga score	Exacerbated	8	0.41 (0.07-3.32)	2	2.43 (0.30-13.36)
	Non exacerbated	69	2.43 (0.30-13.36)	7	0.41 (0.07-3.32)
Transcutaneous oxygen saturation of hemoglobin	Normal (>95%)	53	0.26 (0.01-1.71)	9	3.86 (0.58-89.85)
	Mild (91-95%)	19	2.97 (0.44-69.6)	1	0.34 (0.01-2.25)
	Moderate (85-90%)	2	-	0	-
	Severe (<85%)	2	-	0	-

TNF-α = tumor necrosis factor; FVC = forced ventilator capacity. Statistical analysis performed by χ^2 test and Fisher's exact test.

was a protective factor for early onset of pulmonary symptoms (OR=12.26, 95%CI=0.08-0.89), and for the first *P. aeruginosa* (under 12 months) (OR=12.15, 95%CI=0002-0007), however, the same allele was a risk factor for worst values of transcutaneous oxygen saturation (values between 91 to 95%) (OR=7.01, 95%CI=1.14-157.4).

Discussion

The difficulty in establishing association between *CFTR* genotype and CF phenotype is well known in the scientific literature. Currently, the search for modifier genes of the phenotype has gained prominence, and allowed a better understanding of the clinical phenotype of the CF patients.

Cystic fibrosis population

There was a predominated adolescent population, with a mean age of 9.71 (± 6.6) years. In age group distribution, the most patients were in the group between zero and ten years (69.38%). By the inclusion of only patients with severe mutations, the observed data may be associated with severe prognosis, with low life expectancy associated with severe *CFTR* mutations.

Table 6. Association of the -238G>A e -308G>A polymorphisms of the *TNF-α* gene with clinical variables with numerical distribution

Clinical variable	-238G>A genotype	N (%)	Mean	Standard deviation	*p*-value
Age at diagnosis (months)	GG	34 (77.27)	30.23	40.11	1.0
	GA	10 (22.72)	27.09	39.39	
Onset of pulmonary symptoms (months)	GG	32 (78.04)	22.22	44.31	1.0
	GA	9 (21.95)	13	18.43	
Onset of digestive symptoms (months)	GG	20 (74.04)	22.45	29.39	0.126
	GA	17 (62.96)	5	3.31	
Forced expiratory volume in first second of the FVC	AG	6 (19.35)	75	33.97	0.4973
	GG	25 (80.64)	73	28.39	
Shwachman-Kulczycki score	AG	4	85	12.90	1.0
	GG	20	74	13.38	
Clinical variable	-308G>A genotype	N (%)	Mean	Standard deviation	*p*-value
Age at diagnosis (months)	GG	7 (15.90)	12.71	13.04	GG vs GA = 1.0
	GA	8 (18.18)	39.75	66.42	GG vs AA = 1.0
	AA	29 (65.90)	31.03	33.97	**GA vs AA = 0.031**
Onset of pulmonary symptoms (months)	GG	7 (17.07)	9.28	15.14	GG vs GA = 1.0
	GA	8 (19.51)	31.85	62.68	GG vs AA = 1.0
	AA	26 (63.41)	18.30	36.96	GA vs AA = 0.226
Onset of digestive symptoms (months)	GG	3 (11.11)	4.33	1.52	GG vs GA = 1.0
	GA	4 (14.81)	23.25	25.07	GG vs AA = 1.0
	AA	20 (74.07)	18.9	28.54	GA vs AA = 1.0
Forced expiratory volume in first second of the FVC	GG	3 (9.68)	85	13.45	GG vs GA = 1.0
	GA	4 (12.90)	82	48.39	GG vs AA = 1.0
	AA	24 (77.42)	71	27.10	GG vs GA = 0.253
Shwachman-Kulczycki score	GG	4 (16.16)	74	11.81	GG vs GA = 1.0
	GA	4 (16.16)	62	14.14	GG vs AA = 1.0
	AA	16 (66.66)	81	15.62	GA vs GG = 1.0

TNF-α = tumor necrosis factor; FVC = forced ventilator capacity; N = number of patients. Positive *p*-value is in bold. Statistical analysis performed by Kruskal-Wallis test and T-student test.

However, life expectancy, quality of life and prognosis in CF have improved. One of the factors that have been blamed for this fact is the systematic care of patients in specialized centers [28]. In the 30s, when the CF was described, 80% of children died in the first years of life. In 1980, the survival rate increased to 20 years, reaching 28 in the 90s and in 2001 reached 32 years old [29].

Regarding sex, a uniform distribution was observed, with 51.02% of women, a fact associated with autosomal recessive inheritance. However, the literature reports a slight predominance of males compared to females, with the increasing of the patients age, and in Brazil, the same was observed with males with values around 60% [30, 31]. Results with different data of our study. However, in our study, only patients with two mutations of Class I and II were included. The lowest prevalence in females may occur by female vulnerability to certain clinical characteristics, such as, the occurrence of diabetes mellitus.

The mean of age at diagnosis (2.47 years) was higher than that found by Dorfman et al. [32] (0.36 years) in a group of 611 patients F508del homozygous. The mean of age for the first infection by the *P. aeruginosa* (3.17 years) was minor than that described by Dorfman et al. [32] (7.5 years). The difference can be explained by the age of diagnosis of the Dorfman et al. [32] to be minor than the present study, sug-

Table 7. Association of -308G>A polymorphism (genotypes) of the *TNF-α* gene with clinical variables of cystic fibrosis patients

Clinical variable	Groups	Genotype distribution					
		G/G	OR (95%CI)	G/A	OR (95%CI)	A/A	OR (95%CI)
Age at diagnosis	<12 months	5	2.13 (0.37-17.39)	3	0.32 (0.06-1.49)	19	1.45 (0.42-5.03)
	13 to 36 months	1	0.81 (0.03-6.80)	3	3.29 (0.53-18.61)	4	0.44 (0.08-2.23)
	>36 months	1	0.41 (0.02-3.18)	3	1.44 (0.25-6.98)	9	1.17 (0.30-5.18)
Onset of pulmonary symptoms	<12 months	4	0.41 (0.07-2.55)	8	3.31 (0.45-82.17)	22	2.92 (0.53-17.87)
	13 to 36 months	3	6.00 (0.85-41.44)	1	0.63 (0.024-5.17)	3	0.36 (0.06-1.97)
	>36 months	0	-	0	-	5	-
	No clinical symptom	0	-	0	-	1	-
Onset of digestive symptoms	<12 months	3	0.33 (0.04-3.32)	7	-	21	**5.98 (1.06-49.68)**
	13 to 36 months	1	2.50 (0.08-30.47)	0	-	3	1.36 (0.13-39.32)
	>36 months	1	2.50 (0.08-30.47)	0	-	3	1.36 (0.13-39.32)
Age for the first infection by *Pseudomonas aeruginosa*	<12 months	7	-	7	**10.2 (1.86-84.09)**	1	**0.08 (0.0003-0.07)**
	13 to 36 months	0	-	2	1	11	1
	>36 months	0	-	0	-	14	-
	No clinical symptom	0	-	0	-	5	-
Forced expiratory volume in first second of the FVC	Normal (>80%)	2	2.25 (0.16-72.3)	2	1.07 (0.10-11.6)	11	0.54 (0.10-3.78)
	Mild (60-80%)	1	1.48 (0.04-22.1)	1	0.95 (0.32-10.58)	6	0.84 (0.13-7.65)
	Moderate (40-60%)	0	-	0	-	3	-
	Severe (<40%)	0	-	1	1.87 (0.06-22.97)	4	1.19 (0.12-34.39)
Shwachman-Kulczycki score	Excellent (86-100)	2	1.82 (0.17-15.96)	2	0.74 (0.08-5.15)	3	0.68 (0.10-4.29)
	Good (71-85)	2	1.82 (0.17-15.96)	1	0.32 (0.01-2.91)	4	1.48 (0.23-10.16)
	Mild (56-70)	1	-	0	-	0	-
	Moderate (41-55)	0	-	4	4.04 (0.60-31.01)	4	1 (0.17-5.97)
	Severe (≤40)	0	-	0	-	1	-
Kanga score	Exacerbated	0	-	2	4.19 (0.41-35.52)	3	0.62 (0.08-5.81)
	Non exacerbated	6	-	5	0.24 (0.028-2.42)	27	1.62 (0.17-12.31)
Transcutaneous oxygen saturation of hemoglobin	Normal (>95%)	6	-	6	2.59 (0.33-66.37)	19	0.15 (0.0006-1.05)
	Mild (91-95%)	0	-	1	0.51 (0.02-4.10)	9	4.99 (0.69-122.5)
	Moderate (85-90%)	0	-	0	-	1	-
	Severe (<85%)	0	-	0	-	1	-

TNF-α = tumor necrosis factor; FVC = forced ventilator capacity. Positive *p*-value is in bold. Statistical analysis performed by χ^2 test and Fisher's exact test.

gesting that treatment of those patients started before our sample, what may have delayed the colonization by *P. aeruginosa*.

Analysis of polymorphisms in TNF-α gene

Analysis of polymorphism -238G>A in TNF-α gene: Of the 49 CF patients, 79.59% were homozygous for the G allele, and 20.41% heterozygous. The allele frequency found was 0.90 and 0.10 for the G and A allele, respectively. Buranawuti et al. [20] analyzed 101 children and 115 adults with CF, and found the GG genotype in most individuals analyzed (91.1%), and, as in our study, no patients with the AA genotype was found. Yarden et al. [16] studied 180 CF patients with F508del/F508del genotype and the GG genotype was found with higher frequency (88.2%), being the AA genotype detected in 1.2% of the patients.

Age at diagnosis, onset of pulmonary and digestive symptoms were not associated with the polymorphism. It was expected that the age at diagnosis and onset of symptoms occurred in most patients within the first year of life, which denotes a worse prognosis and more severe presentation of the disease, and that these, were associated with more severe allele, however, there was no association

For the first isolation of *P. aeruginosa* was not found association with the -238G>A genotype of *TNF-α* gene. This data is in accordance with Yarden et al. [16] study (p=0.64). As the most patients had early diagnosis, it was expected that the age of first *P. aeruginosa* infection was delayed, being that the early treatment could delay the infection. But, this was not observed in our study.

Table 8. Association of -308G>A polymorphism (alleles) of the *TNF-α* gene with clinical variables of cystic fibrosis patients

Clinical variables	Groups	Allelic distribution			
		G allele	OR (95%CI)	A Allele	OR (95%CI)
Age at diagnosis	<12 months	13	1.01 (0.39-2.69)	41	0.99 (0.37-2.56)
	13 to 36 months	5	1.56 (0.44-5.06)	11	0.64 (0.20-2.29)
	>36 months	5	0.69 (0.21-2.05)	21	1.45 (0.48-4.87)
Onset of pulmonary symptoms	<12 months	16	0.75 (0.26-2.25)	52	1.33 (0.45-3.79)
	13 to 36 months	7	**3.81 (1.13-12.97)**	7	**0.26 (0.08-0.89)**
	>36 months	0	-	10	-
	No clinical symptom	0	-	2	-
Onset of digestive symptoms	<12 months	13	0.80 (0.22-3.29)	49	1.25 (0.30-4.48)
	13 to 36 months	2	1.22 (0.16-6.44)	6	0.82 (0.16-6.43)
	>36 months	2	1.22 (0.16-6.44)	6	0.82 (0.16-6.43)
Age for the first infection by *Pseudomonas aeruginosa*	<12 months	21	**66.77 (15.18-482.7)**	9	**0.15 (0.002-0.07)**
	13 to 36 months	2	**0.15 (0.02-0.62)**	24	**6.69 (1.62-45.66)**
	>36 months	0	-	28	-
	No clinical symptom	0	-	10	-
Forced expiratory volume in first second of the FVC	Normal (>80%)	6	2.30 (0.52-12.34)	24	0.44 (0.08-1.94)
	Mild (60-80%)	3	1.49 (0.27-6.91)	13	0.67 (0.14-3.70)
	Moderate (40-60%)	0	-	6	-
	Severe (<40%)	0	-	9	-
Shwachman-Kulczycki score	Excellent (86-100)	6	2.41 (0.60-9.84)	8	0.41 (0.10-1.68)
	Good (71-85)	5	1.51 (0.36-6.08)	9	0.66 (0.16-2.77)
	Mild (56-70)	2	-	0	-
	Moderate (41-55)	0	-	12	-
	Severe (≤40)	0	-	2	-
Kanga score	Exacerbated	2	0.87 (0.12-4.20)	8	1.15 (0.24-8.58)
	Non exacerbated	17	1	59	1
Transcutaneous oxygen saturation of hemoglobin	Normal (>95%)	18	**9.24 (1.53-206.1)**	44	**0.11 (0.005-0.66)**
	Mild (91-95%)	1	**0.14 (0.006-0.87)**	19	**7.01 (1.14-157.4)**
	Moderate (85-90%)	0	-	2	-
	Severe (<85%)	0	-	2	-

TNF-α = tumor necrosis factor; FVC = forced ventilator capacity. Positive *p*-value is in bold. Statistical analysis performed by χ^2 test and Fisher's exact test.

In the classification of severity based on FEV_1%, 70.96% of patients were classified as having normal and mild phenotype, and the classification was not associated with the polymorphisms examined, as well the mean values of FEV_1%. The results are in agreement to that reported by Yarden et al. [16], who found no relationship between FEV_1% with the genotype for the polymorphism analyzed (p=0.8).

The distribution of genotypes for the -238G>A polymorphism of *TNF-α* gene in the sample compared to the classification obtained by the Shwachman-Kulczycki score and the mean values, showed no significant association.

The score Kanga showed that at the time of the examination the most part of the patients (88.37%) were not in exacerbation, and that 81.57% of these patients were homozygous for the G allele, however, the association was not positive. The severity classification by transcutaneous oxygen by hemoglobin showed that 72.09% of patients were normal, and that the majority of these (70.96%) were homozygous for the G allele, however, the association was not positive.

Based on these results, in the evaluated sample, there was no association between the polymorphic variant (-238G>A) and CF severity, as proposed by Yarden et al. [16].

Analysis of polymorphism -308G>A in TNF-α gene: Of the 49 patients studied, 14.28% were GG homozygotes, 67.35% AA homozygotes and

18.36% GA heterozygotes. The allele frequencies were 0.77 and 0.23 for allele A and G, respectively, data contrary to reported in the literature. The G allele in other studies showed higher frequency than the A allele, being the frequency of G allele of 0.7, 0.75 and 0.81 (only patient AA homozygote), respectively, for the study of 53, 261 and 53 CF patients [15, 33, 34]. The variation can be explained by admixture population analyzed and the kind of colonization occurred in the our country.

Age at diagnosis (except the minor time for the diagnostic for G/A genotype vs AA genotype) and onset of pulmonary symptoms (for genotypic analysis) showed no association. However, the A allele, previously described as risk factor for the severe pulmonary disease [15], was associated with increased risk of early digestive disease (under 12 months) and had lower odds ratios for late-onset pulmonary disease, confirming the previously reported findings.

The colonization by *P. aeruginosa* was associated with the -308G>A genotype of *TNF-α* gene, being the AA genotype a protective factor for early colonization and GA a risk. The finding is not in agreement with that described by Arkwright et al. [34] and Yarden et al. [16] studies in homozygotes patients (F508del) from Belgium and Czech Republic. The G allele was a risk factor for early colonization and the A allele a protector factor. This data were also not previously reported in the scientific literature referenced.

In the classification of severity based on FEV_1%, 74.19% of patients were classified as normal and mild pulmonary phenotype. It was expected that the A allele of the -308G>A polymorphism of *TNF-α* gene was associated with greater clinical severity. The majority (73.91%) of patients with greater than of 80% of predicted FEV_1 had the AA genotype. The A allele showed a higher odds ratio for worse saturation, and the G allele, higher odds ratio for better saturation. Thus, the data confirmed the greater severity of the lung disease associated with the A allele. By the comparison between the mean of FEV_1% was held, for the -308G>A genotype of *TNF-α* gene, there was no association. Hull and Thompson [15] demonstrated that the mean of FEV_1% in patients with GA genotype was lower than in GG group, which reinforces the hypothesis of these authors that the A allele, is associated with severe lung disease. Schmitt-Grohe et al. [33] did not observe the same differences compared to FEV_1%, considering -308G>A genotype in the *TNF-α* gene, the same occurred in the study of Yarden et al. [16].

For the Shwachman-Kulczycki score no association was described, the same was observed in other studies [15, 33]. For the Kanga score, 88.37% of the patients without exacerbation, 71.05% were homozygotes for the A allele. However, no significant association was observed.

For transcutaneous hemoglobin oxygen saturation, of 72.09% of the patients considered normal, 63.33% were homozygotes for A allele. This results are contrary to the Hull and Thompson [15] in which the A allele was associated with severe disease. However, it is consistent with other studies [16, 33].

Conclusion

The study of polymorphisms in CF modifier genes present importance to promote greater understanding of the factors that influence the clinical variability of the disease. In the present study, for the two polymorphisms of the *TNF-α* gene, only the -308A>G polymorphism was associated with clinical severity. Thus, it emphasizes the need for more studies for polymorphisms and other possible modifiers genes in CF, considering the large number of studies with conflicting results, and models of sampling.

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Disclosure of conflict of interest

None.

Abbreviations

ACE, angiotensin I converting enzyme; *ADRA2A*, adrenoceptor alpha 2A; *ADRB2R*, Beta-2 adren-

ergic receptor; ARMS-PCR, amplification refractory mutation system; BMI, Body mass index; CF, Cystic Fibrosis; *CFTR*, Cystic Fibrosis Transmembrane Regulator; *COX-2*, cytochrome c oxidase; FEV_1, Forced Expiratory Volume in the first second; FVC, forced vital capacity; *GCLC*, Glutamate-cysteine ligase; *GSTP1*, Glutathione S-transferase pi 1; *GSTM1*, Glutathione S-transferase mu 1; *IFRD1*, Interferon-related developmental regulator 1; *MBL-2*, Mannose-Binding Lectin (Protein C) 2; PCR, Polymerase chain reaction; RFLP, restriction fragment length polymorphism; *TCF7L2*, transcription factor 7-like 2 (T-cell specific, HMG-box); *TGF-β1*, Transforming Growth Fctor, Beta 1; *TNF-α*, tumor necrosis factor; USA, United States of America.

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