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ADRA2A IS A CYSTIC FIBROSIS MODIFIER GENE

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Abstract-

Background: Cystic fibrosis (CF) is an autosomal disease with characteristics of a complex disease. Understanding *ADRA2A* polymorphisms are important to elucidate clinical variability that is encountered in inflammatory diseases including CF, for which diabetes is an important comorbidity beyond the primary inflammatory pulmonary disease.

Method: We included 176 CF patients. The rs553668 and rs10885122 *ADRA2A* gene polymorphisms were screened by ARMS-PCR. A genotypic comparison was performed with 27 CF clinical variables and *CFTR* mutations.

Results: Clinical associations were found with the categorical variables: race [rs553668 polymorphism without taking the *CFTR* gene into account (p= 0.002); haplotype group, without taking the *CFTR* gene into account (p= 0.014)], meconium ileus [rs553668 polymorphism without taking the *CFTR* gene into account (p= 0.030) and patients with two *CFTR* mutations (p= 0.0012)] and BMI [rs553668 polymorphism in patients with two *CFTR* mutations (p= 0.014)]. The association with numerical data was positive for age of diagnosis [rs553668 polymorphism without taking *CFTR* mutations into account (p= 0.022)]; the Bhalla score [rs553668 polymorphism in patients with two *CFTR* mutations (p= 0.014)]; and the Shwachman-Kulczycki score [rs553668 polymorphism (p= 0.008) and haplotype (p= 0.050) in patients with two *CFTR* mutations].

Conclusion: The rs553668 and rs10885122 *ADRA2A* gene polymorphisms are modifiers of CF severity.

Keywords- Cystic fibrosis, polymorphisms, ADRA2A, CFTR, genotype, phenotype, variability, lung disease, modifier gene

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Background

Cystic fibrosis (CF) is an autosomal disease that has characteristics of a complex disease. CF clinical modulations are associated with the environment and modifier genes [1-3]. The environment is a factor that cannot be controlled for statistical analysis. However, modifier genes are study targets that enable greater understanding of clinical disease variability, especially in regard to the lung disease.

Our group studied CF severity in association with several modifier genes including polymorphisms in the following genes: *MBL-2, TGF -β1*, *CD14* [4], *GSTM1*, *GSTT1* [5], *ACE* [6], *ADRB2* [7] and *TCF7L2* [8]. These polymorphisms are associated with clinical variables including lung and digestive disease.

In CF, clinical variability is associated with clinical variables; however, much remains to be analyzed, and modifier genes that are associated with the immune response have been a target for studying genetic modulation and identifying new therapeutics [1-3]. Among these, the 2-adrenergic receptor alpha (*ADRA2A*) gene has been studied.

The *ADRA2A* gene region 10q24-q25 encodes a protein with 450 amino acids, which is known as alpha-2A adrenergic receptor (α2-AR). Adrenergic receptors are part of a family of G protein-coupled receptors that are stimulated by catecholamines such as epinephrine and norepinephrine. Generally with stimuli, adrenergic receptors activate G proteins that will stimulate enzymes such as adenylate cyclase and phospholipase C to induce second messenger production including cyclic adenosine 5'-monophosphate (cAMP) or inositol 1,4,5-triphosphate (IP3), diacylglycerol (DAG) and Ca^{2+}. Conversely, the α2-AR G-protein coupled receptor inhibits adenylate cyclase and subsequent cAMP formation [9,10].

The α2-AR is found in adrenergic cells and is highly expressed in sympathetic nerve center outputs, the cerebral cortex, the hippocampus, the septum, the amygdaloid, hypothalamic nuclei, the umbilical cord, the spine, pancreatic islets, platelets and immune cells (macrophages, polymorphonuclear cells and T lymphocytes) [10-13]. The α2-AR protein reduces sympathetic tone and norepinephrine levels, blood pressure and heart rate, thus providing sedation and analgesia. The α2-AR protein is also involved in glucose and

lipid metabolism, body temperature regulation, platelet aggregation, proinflammatory cytokine production, cognition and behavior [14,15].

Studies have correlated α2-AR receptor expression with diabetes mellitus risk, pulmonary inflammatory process intensity during bacterial infections and allergic asthma-related bronchoconstriction [15-17]. In this context, studying *ADRA2A* polymorphisms is important to elucidate clinical variability in inflammatory diseases including CF, for which diabetes is an important comorbidity beyond the inflammatory pulmonary disease.

The aim of this study was to investigate the association of 27 CF clinical variables with *ADRA2A* polymorphisms.

Methods

Cross-sectional studies were conducted in a university center for CF care between 2012 and 2013. CF diagnosis was confirmed in patients with two tests that gave sweat sodium and chloride values greater than 60 mEq/L.

In a cohort of patients we identified two mutations in the Cystic Fibrosis Transmembrane Regulator (*CFTR*) gene. No patient had received the neonatal CF screening test.

In total, 215 patients were selected for the study. Of these, 176 were included, and 39 patients who did not have clinical data for statistical analysis and had not signed the consent form were excluded.

Patient DNA was obtained by phenol-chloroform extraction.

The DNA concentration used for analysis was 50 ng/mL, which was evaluated using a GE NanoVue™ spectrophotometer (GE Healthcare Biosciences, Pittsburgh, USA).

Clinical Markers of Disease Severity

The following clinical severity markers were employed: clinical scores (Shwachman-Kulczycki, Kanga and Bhalla) [18], body mass index (BMI) (for patients older than 19, the BMI= weight/(height)^2 formula was used; for the remaining patients, the WHO ANTHRO (children under 5 years old) and WHO ANTHRO PLUS (children 5-19 years old) programs were used [19,20]), patient age (≤ 154 and > 154-month-old age groups), age at diagnosis (sodium and chloride levels with altered perspiration ≤ 24 and > 24 months), first clinical symptoms (digestive ≤ 3 and > 3 months; pulmonary ≤ 6 and > 6 months), 1st *Pseudomonas aeruginosa* colonization (≤ 31 and > 31 months), sputum microorganism presence (*P. aeruginosa* mucoid (PAM) and non-mucoid (PANM), *Achromobacter xylosoxidans, Burkolderia cepacia* and *Staphylococcus aureus*), transcutaneous hemoglobin oxygen saturation and spirometry.

Spirometry was performed in patients who were at least 7 years old with the CPFS/D spirometer (MedGraphics, Saint Paul, Minnesota, USA). Data were recorded using PF BREEZE software version 3.8B for Windows 95/98/NT, and the following markers were included: forced vital capacity [FVC(%)], forced expiratory volume in the first second [FEV_1(%)], the ratio between FEV_1 and FVC(%) [FEV_1/FVC(%)], and forced expiratory flow between 25 and 75% of the FVC [FEF_{25-75}%].

The analyzed comorbidities were nasal polyps, osteoporosis, meconium ileus, diabetes mellitus, and pancreatic insufficiency.

This study was approved by the Institutional Ethics Committee from the University of Campinas Medical Faculty (#528/2008), and all of the patients signed a consent form before beginning the study.

CFTR Mutation Determination

CFTR mutation determination was performed by polymerase chain reaction (PCR) (F508del mutation) and the fragment length polymorphism method (G542X, R1162X, R553X, G551D and N1303K). Some mutations in CF patients were obtained by sequencing or MLPA (multiplex ligation-dependent probe amplification) analysis, S4X, 2183A>G, 1717G>A and I618T. For sequencing and MLPA, we used the same MegaBACE 1000® (GE Healthcare Biosciences, Pittsburgh, USA). The *CFTR* genotype was used as a correction factor for statistical analysis. All of the mutations identified were included in class one, two or three of the *CFTR* gene. Other mutations that were as class IV (P205S e R334W) were not included in the statistical analysis. For more details see the session results.

ADRA2A Polymorphism Determination

To determine the genotype of the rs553668 and rs10885122 polymorphisms in the *ADRA2A* gene, the amplification refractory mutation system (ARMS-PCR) was used because it can specifically determine single sequence polymorphisms with the same sensibility and specificity as restriction fragment length polymorphism analysis (RFLP) [21]. To assess rs553668 polymorphisms the following primers were used: 3′- CCA AGG CCA GGA TTT CAA CA -5′ (common primer), 3′- CCC AAC TCT CTC TCT CTT TTT TG -5′ and 3′- CCC AAC TCT CTC TCT CTT TTT TA -5′ (specific primers). The following primers were used to assess rs10885122 polymorphisms: 3′- TTC CCT GCT CAG AAA CAT CC -5′ (common primer), 3′- GTA TCA ACA GGT TTC ACA AGG -5′ and 3′- GTA TCA ACA GGT TTC ACA AGT -5′ (specific primers).

The rs553668 PCR fragment was 152 base pairs and the rs10885122 PCR fragment was 221 base pairs.

Polymorphism PCR was performed with bidistilled water, 10x Taq buffer with $(NH_4)_2SO_4$, $MgCl_2$ (25 mM), dNTP (25 mM each), primers (0.2 pmol of each primer), Taq polymerase (5U) and genomic DNA (50 ng/mL).

The annealing temperatures for rs553668 and rs10885122 were 56°C and 60°C, respectively.

The reaction was analyzed on a polyacrylamide gel (12%) at 180 V for 4 hours. The gel was stained in ethidium bromide solution and visualized on the Typhoon™ scanner (GE Healthcare, Wisconsin, USA).

Statistical Analysis

Statistical analysis was performed with Statistical Package for the Social Sciences (SPSS) software v.17.0 (version 17, SPSS Inc., Chicago, IL), Epi Info v.6.0 [22] and R version 2.12 (Comprehensive R Archive Network, 2011). Sample statistical power calculations were performed with GPower 3.0.3.1 software [23]; statistical power for the analysis was above 80%.

The data were compared using the c^2 and Fisher exact tests for categorical variables and the Mann-Whitney and Kruskal-Wallis tests for numerical variables.

The rs553668 and rs10885122 polymorphisms were compared directly with the variables according to results session. All of the associations for the analyzed haplotypes were performed considering whether the genotypes were heterozygous for either polymorphism according to results session. For additional details, consult [Fig-1].

Because of the high standard deviation in the patient data distribu-

tion several variables were categorized into classes based on time (short or long) and severity (minor or severe) including patient age, age at diagnosis, onset of pulmonary and digestive symptoms and time before *P. aeruginosa* isolation.

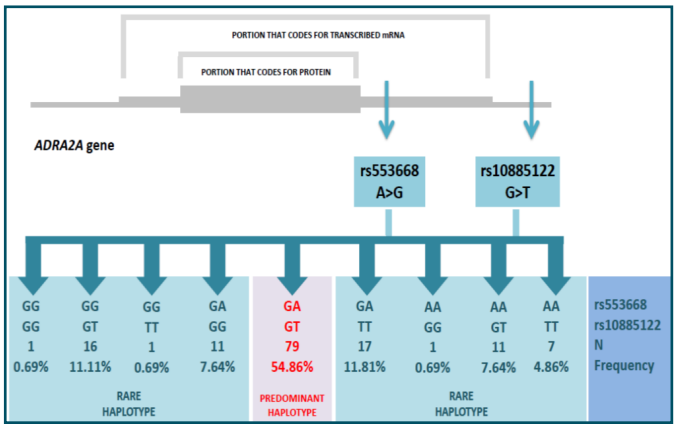

Fig. 1- Haplotype groups for the rs10885122 and rs553668 *ADRA2A* gene polymorphisms.

[Fig-1] demonstrates the high prevalence for the heterozygous *ADRA2A* gene rs10885122 and rs553668 polymorphism haplotype and polymorphism localizations in the *ADRA2A* gene. All of the possible polymorphism combinations are demonstrated; patient number (N) and frequency (%) in each group.

To avoid spurious data because of multiple problematic tests [24], the significance level α was adjusted using the Bonferroni correction test ($\alpha_{corrected}$= 0.05/number of tests).

The analyses were performed using two cohorts. The first cohort included all of the CF patients (176 patients), and second cohort contained patients with two *CFTR* gene mutations (78 patients). The latter was the main cohort that was used to analyze modifier gene influence associated with CF clinical variations.

Results

In total, 176 CF patients were included in the study. All of the CF patient clinical features are demonstrated in [Table-1]. Characterization of the *ADRA2A* gene polymorphism and *CFTR* mutation genotypes among the CF patients is shown in [Table-2]. The *ADRA2A* gene rs553668 and rs10885122 polymorphism haplotype groups are described in [Table-3]. There was prevalence to the GA/GT group, which included 79 (54.86%) patients.

The association of the *ADRA2A* gene rs10885122 and rs553668 polymorphisms with CF patient clinical variables distributed by *CFTR* mutation and haplotype are described in [Table-4].

The association of CF severity with rs553668 polymorphism and *ADRA2A* gene haplotype in regard to the categorical variable with a positive corrected p-value is demonstrated in [Table-5].

The association of age of diagnosis and the Shwachman-Kulczycki score with *ADRA2A* gene rs553668 polymorphism and CF haplotype is demonstrated in [Table-6].

Associations were found with the categorical variables: race [rs553668 polymorphism without taking the *CFTR* gene into account (p= 0.002); haplotype group without taking the *CFTR* gene into account (p= 0.014)], meconium ileus [rs553668 polymorphism without taking the *CFTR* gene into account (p= 0.030), and patients with two *CFTR* mutations (p= 0.0012)], BMI [rs553668 polymorphism in patients with two *CFTR* mutations (p= 0.014)], which are described in [Table-4] and [Table-5]. The association with numerical data was positive for age of diagnosis [rs553668 polymorphism without taking the *CFTR* mutation into account (p= 0.022)]; the Bhalla score [rs553668 polymorphism in patients with two *CFTR* mutations (p= 0.014)]; and the Shwachman-Kulczycki score [rs553668 polymorphism (p= 0.008) and haplotype (p= 0.050) in patients with two *CFTR* mutations].

Table 1- Clinical features of Cystic Fibrosis patients included in the study

Variable	mean ± SD (range)
Sex - male	48% (86)#
Caucasian	91.5% (161)#
Age	208.64 ± 13.85 months (7-833 months)*
BMI - thinness and accentuated thinness	20.69% (36)#
One Class I, II or III identified mutation	27.93% (49)#
Two Class I, II or III identified mutations	43.31% (76)#
First clinical manifestation	35.44 ± 8.52 months (0-720 months)*
Age at diagnosis	91.93 ± 12.70 months (0-720 months)*
Digestive symptom onset	41.49 ± 9.10 months (0-720 months)*
Pulmonary symptom onset	43.79 ± 9.46 months (0-720 months)*
SpO2	94.99 ± 0.32 (66-99)*
Bhalla score	8.67 ± 0.49 (0-23)*
Kanga score	18.60 ± 0.48 (10-40)*
Shwachman-Kulczycki score	66.14 ± 1.35 (20-95)*
FVC (%)	79.73 ± 2.05 (19-135)*
FEV_1 (%)	71.97 ± 2.39 (17-132)*
FEV_1/FVC (%)	83.95 ± 1.37 (37-137)*
FEF_{25-75}%	59.81 ± 3.11 (5-150)*
Nasal Polyps	19.08% (33)#
Diabetes mellitus	18.50% (32)#
Osteoporosis	16.18% (28)#
Pancreatic insufficiency	80.92% (140)#
Meconium ileus	14.86% (26)#
First isolated P. aeruginosa	104.09 ± 15.36 months (2-180 months)
P. aeruginosa status [1]	55.10% (97)#
P. aeruginosa mucoid status [1]	42.00% (74)#
B. cepacia status [1]	13.60% (24)#
A. xylosoxidans status [1]	10.20% (18)#
S. aureus status [1]	78.40% (138)#

BMI = body mass index; % = percentage; SpO2 = Transcutaneous Hemoglobin Oxygen Saturation, FVC = forced vital capacity; FEV_1 = forced expiratory volume in the first second; FEF_{25-75} = forced expiratory flow between 25 and 75% of FVC. 2. Based on 3 consecutive positive respiratory cultures.

*# Percentage (patient number)**

Continuous variables are expressed as the mean ± SD (range)

Discussion

The modifier gene study allows for a better understanding of the variability of CF severity. Studies on lung disease modifier genes have been rarely performed because the focus in current studies is searching for new drugs that are responsive to specific mutations in the *CFTR* gene.

Our group has analyzed genes associated mainly with the immune system in combination with CF clinical severity [4-8], and we have tried to elucidate how disease variability of the occurs primarily in regard to the pulmonary phenotype.

Thus, the *ADRA2A* gene and its polymorphisms (rs553668 and rs10885122) were selected. These polymorphisms have been associated with diabetes mellitus in previous studies because diabetes mellitus is a CF comorbidity that has increased together with increased life expectancy in CF patients. Moreover, these polymorphisms have importance in the immune system, particularly in response to bacteria that colonize the airways.

The pancreas is richly innervated by sympathetic neurons and nora-

drenaline and adrenaline acts to negatively regulate insulin secretion. Catecholamines stimulate α2-ARs that are present in the pancreatic islet β cell postsynaptic membrane by blocking insulin release and causing hyperglycemia. It has been suggested that increased parasympathetic innervation or an enhanced response of these receptors or both contributes to the deficient secretory response to glucose in type 2 diabetes mellitus (DMII) patients [11]. α2-AR overexpression contributed to DMII development [17]. The rs553668 polymorphism was associated with DMII risk. The A allele was associated with a higher fasting blood glucose value compared with the homozygous G allele, and the same was observed for the rs553668A/rs10885122T haplotype [25], which is associated with DMII, an important CF comorbidity.

Table 2- Genotypic characteristic of ADRA2 gene polymorphisms and CFTR gene mutations among Cystic Fibrosis patients

Gene	Chromosome position	Location	Variation	Genotype			MAF	c²	p*
				G/G	G/T	T/T			
ADRA2, rs10885122	10q25.2	intergenic region	G>T	17 (10.24%)	115 (69.28%)	34 (20.48%)	0.45	26.59	<0.001[1]
				A/A	A/G	G/G			
ADRA2, rs553668	10q25.2	3' untranslated region	A>G	20 (12.99%)	116 (75.32%)	18 (11.69%)	0.49	39.55	<0.001[1]
CFTR mutation genotype		N				Frequency			
F508del/F508del		47				26.70%			
F508del/G542X		12				6.82%			
F508del/R1162X		5				2.84%			
F508del/N1303K		4				2.27%			
F508del/R553X		2				1.14%			
F508del/S4X		1				0.57%			
F508del/1717-1G>A		1				0.57%			
F508del/2184insA		1				0.57%			
G542X/R1162X		1				0.57%			
G542X/I618T		1				0.57%			
G542X/2183A>G		1				0.57%			
R1162X/R1162X		1				0.57%			
F508del/duplication of exons 6b to 16		1				0.57%			
711+1G→T/622-2A>G		1				0.57%			
3120+1G àA /3120+1G àA		1				0.57%			
3120+1G àA/R1066C		1				0.57%			
G542X/P205S		1				0.57%			
G542X/R334W		1				0.57%			
D110H/V232H		1				0.57%			
R334W/R334W		1				0.57%			
R334W/R1066C		1				0.57%			
F508del/-		39				22.16%			
G542X/-		5				2.84%			
R1162X/-		1				0.57%			
3120+1G àA /-		1				0.57%			
I507V/-		1				0.57%			
TG11-5T1/-		1				0.57%			
-/-		42				23.86%			

*ADRA2 = Adrenergic receptor alpha 2; CFTR = Cystic fibrosis transmembrane conductance regulator; C = Cytosine; T = Thymine; A = Adenine; G = Guanine; < = less than; MAF = minor allele frequency; % = percentage; *p = value for Hardy-Weinberg Equilibrium; N = patient number; (-) CFTR mutation no identified. 1= ADRA2, rs10885122 and rs553668 polymorphisms are not in Hardy-Weinberg Equilibrium in our sample.*

Table 3- rs553668 and rs10885122 polymorphism haplotype groups in the ADRA2 gene

Haplotype[1]	Frequency[2]	Genotype combination[3]	Haplotype group[4]
0	1 (0.69%)	GG/GG	0
1	16 (11.11%)	GG/GT	0
2	1 (0.69%)	GG/TT	0
3	11 (7.64%)	GA/GG	0
4	79 (54.86%)	GA/GT	1#
5	17 (11.81%)	GA/TT	0
6	1 (0.69%)	AA/GG	0
7	11 (7.64%)	AA/GT	0
8	7 (4.86%)	AA/TT	0

1. ADRB2 polymorphism haplotype groups. The number code was used to demonstrate the different groups of ADRB2 polymorphism combinations. 2. Haplotype frequency-patient number (percentage). 3. Genotype groups of rs553668 and rs10885122 ADRB2 polymorphisms, respectively. 4. Haplotype group statistical analysis. # Heterozygote rs553668 and rs10885122 ADRB2 polymorphisms were prevalent and used for all of the associations.

α2-AR expression is decreased in the airway by repeated exposure to allergens and other antigens that are associated with the asthma-associated decline in lung function and increased bronchoconstriction [26]. In the respiratory system, α2-AR inhibits smooth muscle tone by partially blocking glutamate release from the axonal endings. The glutamatergic drive to the vagal preganglionic neurons that innervate the tracheobronchial system promote the bronchoconstriction reflex [16], thus promoting respiratory system changes.

Flierl and colleagues (2007) verified that enzymes involved in catecholamine tyrosine hydroxylase (HT) and dopamine β-hydroxylase (DBH) synthesis in macrophages and polymorphonuclear cells (PMNs) enabled cells to synthesize and degrade adrenaline and noradrenaline. This synthesis occurred by stimulating the lipopolysaccharide (LPS) that is present in bacterial cell walls that is responsible for infectious processes. The catecholamines acted in a paracrine manner to stimulate macrophages and PMN α2-ARs [12].

α2-ARs exert important immunomodulatory effects on the antibacterial pulmonary inflammatory response. LPS-induced norepinephrine levels enhance the expression of receptors that promote proinflammatory cytokine (TNF-α, IL-1, IL-6) production by macrophages and polymorphonuclear cells [15]. While α2-AR stimulation in natural

killer T lymphocytes promotes anti-inflammatory cytokine (IFN-γ and IL-4) synthesis [13]. This exacerbated inflammatory response causes more injuries and a subsequent decline in lung function [15]. This phenomenon explains the common lung infections in CF.

In our study, we associated the rs553668 polymorphism and haplotype analysis with CF clinical variables. This analysis encompassed all of the patients including those with two *CFTR* mutations in classes I, II and III.

Table 4- Association of the rs10885122 and rs553668 polymorphisms in the ADRA2A gene with clinical variables in Cystic Fibrosis patients distributed by CFTR mutation and ADRA2A gene haplotype analysis

Variables	rs10885122				rs553668				Haplotype			
	Without taking *CFTR* mutation into account		Two *CFTR mutations* identified		Without taking *CFTR* mutation into account		Two *CFTR identified* mutations		Without taking *CFTR* mutation into account		Two *CFTR mutations* identified	
	p	pc	p	pc	p	pc	p	pc	P	pc	p	pc
Sex[1]	0.085	0.17	0.132	0.264	0.125	0.25	0.463	0.926	0.616	1	0.207	0.414
Race[1]	0.204	0.408	0.059	0.118	**0.001**	**0.002**	0.296	0.592	**0.007**	**0.014**	0.065	0.13
Age[1]	0.685	1	0.232	0.464	0.055	0.11	0.412	0.824	0.695	1	0.117	0.234
Onset of symptoms[1]	0.512	1	0.477	0.954	0.068	0.136	0.122	0.244	0.663	1	0.685	1
Onset of pulmonary disease[1]	0.801	1	0.953	1	0.102	0.204	0.347	0.694	0.773	1	0.337	0.674
Onset of digestive disease[1]	0.259	0.518	0.488	0.976	0.117	0.234	0.04	0.08	0.194	0.388	0.398	0.796
Diagnosis[1]	0.804	1	0.205	0.41	**0.011**	**0.022**	0.196	0.392	0.872	1	0.411	0.822
BMI[1]	0.402	0.804	0.323	0.646	0.208	0.416	**0.007**	**0.014**	0.835	1	**0.028**	0.056
Bhalla score[2]	0.552	1	0.262	0.524	0.945	1	0.571	1	0.282	0.564	0.042	0.084
Kanga score[2]	0.285	0.57	0.253	0.506	0.837	1	0.955	1	0.293	0.586	0.414	0.828
Shwachman-Kulczycki score[2]	0.194	0.388	0.308	0.616	0.27	0.54	**0.004**	**0.008**	0.043	0.086	**0.025**	**0.05**
Nasal polyposis[1]	0.655	1	0.238	0.476	0.124	0.248	0.177	0.354	1	1	1	1
Diabetes *melittus*[1]	0.821	1	0.82	1	0.633	1	0.736	1	0.538	1	1	1
Osteoporosis[1]	0.662	1	0.941	1	0.92	1	0.9.66	1	0.29	0.58	1	1
Meconium ileous[1]	0.524	1	0.19	0.38	**0.015**	**0.03**	**0.006**	**0.0012**	0.815	1	1	1
Insufficiency pancreatic[1]	0.905	1	0.352	0.364	0.728	0.182	0.637	1	0.204	0.408	1	1
SpO2[2]	0.694	1	0.253	0.506	0.204	0.408	0.139	0.278	0.516	1	0.335	0.67
FVC(%)[2]	0.081	0.162	0.053	0.106	0.569	1	0.595	1	0.034	0.068	**0.019**	**0.038**
FEV_1(%)[2]	0.453	0.906	0.18	0.36	0.515	1	0.515	1	0.2	0.4	0.063	0.126
FEV_1/FVC[2]	0.904	1	0.944	1	0.32	0.64	0.779	1	0.467	0.934	0.417	0.834
FEF25-75%[2]	0.777	1	0.502	1	0.52	1	0.522	1	0.622	1	0.159	0.318
1st *P. aeruginosa*[1]	0.323	0.646	0.44	0.88	0.03	0.06	0.23	0.46	0.708	1	0.249	0.498
P. aeruginosa mucoid[1]	0.212	0.424	0.114	0.228	0.118	0.236	0.444	0.888	0.095	0.19	0.311	0.622
P. aeruginosa no mucoid[1]	0.043	0.086	0.615	1	0.622	1	0.886	1	0.028	0.056	0.437	0.874
A. xylosoxidans[1]	0.324	0.648	0.808	1	0.812	1	0.64	1	0.036	0.072	0.531	1
S. aureus[1]	0.274	0.548	0.76	1	0.761	1	0.268	0.536	0.297	0.594	0.167	0.334
B. cepacia[1]	0.919	1	0.804	1	0.821	1	0.319	0.386	0.648	1	0.365	0.73

ADRA2 = Adrenergic receptor alpha 2; CFTR = Cystic fibrosis transmembrane conductance regulator; BMI = body mass index; SpO2 = Transcutaneous Hemoglobin Oxygen Saturation; FVC - forced vital capacity; FEV_1 - forced expiratory volume in the first second; FEF_{25-75} - forced expiratory flow between 25 and 75% of FVC. p = p-value of statistical tests. pc = p-value of statistical tests corrected by Bonferroni's test. P-values that are significantly different are in bold. 1. Categorical variables-the Fisher test was used. 2. Numerical variables-Student's t-test was used.

Table 5- Association of Cystic Fibrosis severity with the rs553668 polymorphism and ADRB2 gene haplotype in reference to the categorical variable with corrected positive p-values

Without taking CFTR mutation into account								
rs553668	Race				Meconium ileus			
	Caucasian	No Caucasian	OR	95% CI	Presence	Absence	OR	95% CI
G/G	17	1	1.64	0.256-37.48	7	11	**4.36**	**1.42-12.94**
G/A	110	6	**4.09**	**1.24-13.86**	15	100	0.48	0.19-1.22
A/A	14	6	0.13	0.04-0.47	2	18	0.56	0.08-2.31
Patients with two class I, II or III CFTR mutations								
rs553668	Meconium Ileus				BMI			
	Presence	Absence	OR	95% CI	T, AT	NW, OV, O	OR	95% CI
G/G	6	6	**6.85**	**1.67-29.19**	6	6	**6.718**	**1.64-28.63**
G/A	7	41	0.43	0.12-1.57	5	43	**0.18**	**0.05-0.66**
A/A	0	9	-	-	2	6	1.475	0.184-8.09
Haplotype analysis								
Haplotype	Race		OR	95% CI				
	Caucasian	No Caucasian						
GA/GT	55	10	**0.15**	**0.02-0.621**				
Others	77	2	**1**	-				

ADRA2 = Adrenergic receptor alpha 2; CFTR = Cystic fibrosis transmembrane conductance regulator; % - percentage; CI = confidential interval; G = guanine; A = adenine; T = thymine; BMI = body mass índex; OR = odds ratio; T = thinness; AT = accentuated thinness; NW = normal weight; OV = overweight; O = obese. Odds ratio to values with statistical significance is in bold.

In clinical practice, the association of the rs553668 polymorphism and both polymorphism haplotypes with the patient's race is not important, but may be a caveat to the presence of an admixture population in our study. Race is associated with polymorphism distribution, but cannot be a risk factor for CF severity.

No additional diabetes risk factors were observed with the polymorphisms; however, the polymorphism rs553668 GG genotype presented risk factors for diagnosis, minor BMI and higher meconium ileus frequency. The GA genotype demonstrated higher values for the Shwachman-Kulczycki score, and the lowest BMI demonstrated protection.

The time of diagnosis is an important clinical variable because the diagnosis is made earlier with more severe disease. Patients with the GG genotype were included in the group with thinness and accentuated thinness together with meconium ileus presence at birth, which is a severity factor. For this polymorphism, the heterozygote patients were protected against thinness and accentuated thinness and had a better Shwachman-Kulczycki score, which is considered to be a good measure of CF severity [18].

The haplotype that was heterozygous for both polymorphisms was associated with higher Shwachman-Kulczycki scores and FVC(%) values, which may be associated with increased polymorphism homozygosity in the study population, which reduces gravity and is evidenced by the higher Shwachman-Kulczycki score and FVC value.

Conclusion

The CF clinical response was associated with *ADRA2A* polymorphisms, especially for the *CFTR* rs553668 polymorphism and haplotype analysis. The *ADRA2A* polymorphism analysis must still be performed in larger populations with complete *CFTR* mutation characterization, and functional analysis of the ADRA2A protein should also be considered.

With increased life expectancy, modifier genes that are mainly active in CF comorbidities are highlighted; thus, further studies should be conducted.

Modifier genes also have an important secondary role as mediators of therapeutic responses and may be important for their correct function.

List of Abbreviations

%: percentage

$(NH_4)_2SO_4$: Ammonium Sulfate

°C: Celsius

ACE: Angiotensin-converting Enzyme

ADRA2A: 2-Adrenergic Receptor Alpha

ADRB2: Beta-2-adrenergic Receptor

ARMS: PCR - amplification refractory mutation system

BMI: Body Mass Index

cAMP: cyclic adenosine 5'-monophosphate

CD14: Monocyte Differentiation Antigen 14

CF: Cystic Fibrosis

CFTR: Cystic Fibrosis Transmembrane Conductance Regulator

CI: Confidential Iterval

DAG: diacyglycerol

DBH: dopamine β-hydroxylase

DMII: diabetes mellitus type II

DNA: Deoxyribonucleic Acid

dNTP: Deoxyribonucleotide Triphosphates

FEF25-75: Forced Expiratory Flow Between 25 and 75% of FVC

FEV_1: Forced Expiratory Volume in the First Second

FVC: Forced Vital Capacity

GSTM1: Glutathione S-transferase Mu 1

GSTT1: Glutathione S-transferase Tetha 1

HT: catecholamines tyrosinehidroxylase

IP3: inositol 1,4,5-triphosphate

LPS: lipopolysaccharide

MBL-2: Mannose-binding Lectin (protein C) 2

MLPA: Multiplex Ligation-dependent Probe Amplification

mM: Milimolar

N: Number of Patients

PAM: *Pseudomonas aeruginosa*

PANM: *Pseudomonas aeruginosa* no mucoid

PCR: Polymerase Chain Reaction

PMNs: Polymorphonuclear cells

pmoL: Picomol

RFLP: restriction fragment length polymorphism

SpO2: Transcutaneous Hemoglobin Oxygen Saturation

SPSS: Statistical Package for Social Sciences

TCFL2: Transcription Factor 7-like 2

TGFβ-1: Transforming Growth Factor beta 1

WHO: World Health Organization

α2-AR: Alpha-2A adrenergic receptor

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Authors Contributions

LMR/FALM: Made substantial contributions to the conception and design of the manuscript as well as data acquisition, data analysis and data interpretation; were involved in drafting the manuscript or revising it critically for important intellectual content.

JDR: performed the molecular genetic studies and was involved in drafting the manuscript or revising it critically for important intellectual content.

CSB: made substantial contributions to the conception and design of the manuscript as well as data acquisition, data analysis and data interpretation; was involved in drafting the manuscript or revising it critically for important intellectual content; gave final approval of the version to be published.

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