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Original Article

Mannose-binding lectin gene as a modifier of the cystic fibrosis phenotype in Argentinean pediatric patients

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Abstract

Background: There is a considerable variation in the phenotype and course of the disease in cystic fibrosis (CF) even in patients with the same *CFTR* genotype, suggesting that other factors are important for prognosis. Mannose-binding lectin (MBL) has been proposed as one of these factors. We therefore investigated the influence of *MBL2* gene variants on disease severity, age at acquisition of *Pseudomonas aeruginosa*, and survival in CF patients.
Methods: MBL2 variants were studied in 106 Argentinean pediatric CF patients carrying two severe *CFTR* mutations. Clinical phenotype was defined according to the Shwachman score and lung function tests. Age at infection with *P. aeruginosa* and age at death were also recorded.
Results: MBL insufficiency was associated with a 3.5-fold risk of having a severe phenotype (CI 95%: 1.2–10.3, $p = 0.03$). It was also associated with an earlier onset of infection with *P. aeruginosa* ($p = 0.035$). No statistically significant differences were found in FEV_1 and survival.
Conclusions: MBL insufficiency was associated with detrimental progression of the disease. These results together with previous findings suggest that the effect of *MBL2* expression may be a major determinant of the severity of the clinical phenotype in patients with CF.

Keywords: Cystic fibrosis; Mannose-binding lectin; *MBL2*; Modifier gene; Phenotype; Polymorphism

1. Introduction

Cystic fibrosis (CF) is an autosomal recessive multisystemic disease caused by mutations in the gene encoding the Cystic Fibrosis Transmembrane Conductance Regulator (*CFTR*). Although CF is a monogenic pathology, there is considerable variation in the phenotype and course of the disease. Phenotypic variation in CF can be attributed to several sources, including different mutations in the *CFTR* gene, other genetic factors, environmental factors, random events, and interactions between any of these [1]. *CFTR* mutations mainly determine the degree of exocrine pancreatic dysfunction, impaired sweat chloride concentration, and malformation of the male reproductive system. Nevertheless, the severity of lung disease, which is the major cause of morbidity and mortality in CF, is poorly correlated with the *CFTR* genotype. The phenotypic variability is seen even among patients with the same mutations in the *CFTR* gene, strongly suggesting the existence of modulating factors that are important for patient prognosis.

In CF, the lung disease is characterized by a chronic infection with typical pathogens such as *Pseudomonas aeruginosa*, *Staphylococcus aureus*, and *Burkholderia cepacia*, which leads to chronic inflammatory damage of lung tissue and progressive loss of lung function. Therefore, it is important to recognize the factors that influence susceptibility to infection.

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One of the first genes postulated as a CF modifier was the mannose-binding lectin 2 (*MBL2*) gene [2]. *MBL2* encodes mannose-binding lectin (MBL), a protein of the innate immune system that is synthesized by the liver and functions as an opsonin and complement activator. Through the lectin domain, MBL binds to carbohydrate structures on a wide range of bacteria, viruses, fungi, and parasites. Thereby, MBL activates the complement pathway lectins with subsequent opsonization or direct lysis of the target [3]. Although MBL is a serum protein, it accumulates in the lungs during acute inflammation in quantities sufficient to promote phagocytosis and activate complement [4]. MBL is biologically active in the oligomerized form. In the general population, levels of oligomerized MBL in the blood vary dramatically due to the presence of certain variants in the *MBL2* gene [5]. The presence of the X allele at position −221 of the *MBL2* promoter produces a lower amount of circulating MBL than that found in the presence of the Y allele. In addition, missense mutations in exon 1 abolish the assembly of the protein into its biologically active form [6–8]. Such mutations include substitution of glycine for aspartic acid at codon 54, glycine for glutamic acid at codon 57, and arginine for cysteine at codon 52. These allelic variants are called *B*, *C*, and *D*, respectively, and are usually collectively referred to as 0 (zero) alleles, while the wild-type allele is labeled *A*. Each genetic variant reduces the amount of functional MBL between 5 and 10 times [9]. It has been shown that low levels of MBL in the blood are associated with susceptibility to infections in various clinical settings [10].

Taking into account that lung infections are the main cause of morbidity and mortality in CF, *MBL2* has been proposed as a modulator of clinical severity in this disease. Assessing different markers of clinical severity, several authors have investigated the role of MBL deficiency as a modifying factor in CF [2,11–13]. Although the results of these studies have not been entirely consistent, according to a recent meta-analysis the available evidence suggests that MBL insufficiency is associated with the severity of CF lung disease [14]. Nevertheless, studies in pediatric populations have not been able to clearly demonstrate the influence of MBL on lung phenotype [15–17]. To date, the vast majority of association studies of MBL and CF have been performed in populations of developed countries of North America and Europe [2,11,12,15–19] and little is known about populations in developing regions, such as Latin America [20]. In these populations, there is a higher proportion of people with low incomes and lack basic services, affecting access to expensive medications, nutritional status, and adherence to treatment.

The aim of this study was to assess the influence of structural and promoter variants in *MBL2* on disease severity, age at acquisition of *P. aeruginosa*, and survival in Argentinean children with cystic fibrosis.

2. Materials and methods

2.1. Study population and data collection

A total of 106 CF patients carrying two severe mutations in the *CFTR* gene were retrospectively evaluated. All patients were diagnosed and followed-up at the Pulmonology Department of a public tertiary-care pediatric center, Hospital de Pediatría "Prof. Dr. Juan P. Garrahan," in Buenos Aires, Argentina. Among the 106 patients, 10 sibling pairs were included. Measurements of forced expiratory volume in one second (FEV_1) and forced vital capacity (FVC) were collected. Age at first positive culture of *P. aeruginosa* and age of death were recorded. Repeated FEV_1 measurements at 7, 8, 9, and 10 years of age were available in 28 patients.

CFTR mutations had been previously determined by molecular methods routinely used at the Laboratory of Molecular Biology of the same hospital (PCR for ΔF508 and Oligonucleotide Ligation Assay–Cystic Fibrosis Genotyping Assay; Celera-Abbott for 32 *CFTR* mutations). In order to exclude variables that could affect the disease phenotype, all patients included in this study had pancreatic insufficiency (PI) and carried severe *CFTR* mutations on both alleles. Patients reported as having PI but carrying at least one *CFTR* mutation typically associated with pancreatic sufficiency were excluded. Of the 106 patients, 75 were homozygous for the severe mutation ΔF508, 27 were heterozygous for ΔF508 together with another severe mutation, and four patients carried two severe non-ΔF508 mutations.

A written informed consent approved by the Ethics Committee of the Hospital was obtained from the parents of each participant.

2.2. MBL2 genotyping

DNA samples extracted from peripheral blood leukocytes by standard procedures were used to characterize *MBL2* genetic variants. Genotyping was performed using PCR restriction fragment-length polymorphism (PCR-RFLP) assays as described elsewhere [21]. Briefly, *Ban*I and *Hha*I restriction enzymes were used for analysis of structural variants and *Sac*II enzyme was used for promoter variants. The resulting fragments were analyzed on 3% Agarose 1000 gel (Invitrogen) stained with ethidium bromide.

Patients with genotypes associated with a high production of MBL (YA/YA, YA/XA) were grouped as MBL-sufficient (MBL-Suf) and patients with genotypes associated with an intermediate and low production of MBL (XA/XA, YA/0, XA/0, 0/0), as MBL-insufficient (MBL-Ins), in a similar approach as described by Yarden et al. [12].

2.3. Clinical phenotype

Lung function testing involves voluntary breathing maneuvers that are usually not possible in children until 5–6 years of age. Therefore, in our study, only patients between 5 and 9 years of age (n = 66) were considered for association analysis of *MBL2* with disease phenotype in order to obtain a homogeneous group with as many lung function studies as possible. The clinical phenotype of these patients was defined as mild, moderate, and severe based on the Shwachman score [22] and lung function studies when available (Table 1).

2.3.1. Shwachman score

The Shwachman score was calculated by pediatric pulmonologists with expertise in CF. This score is divided into four domains, namely: General activity, physical examination, nutrition, and radiological findings, each having five possible subscores according to the degree of impairment. The scores of the four domains are summed to obtain the final score, from which the condition of the patient is categorized as excellent (86–100), good (71–85), average (56–70), poor (41–55), or severe (≤40) [23].

2.3.2. Lung function test

Testing was performed on the Vitalograph® spirometer at the Lung Function Laboratory of the Pulmonology Department in patients over 5 years of age. Forced vital capacity (FVC, FEV_1, and FEF 25–75%) and flow-volume curves were done following the American Thoracic Society guidelines [24]. FEV_1 was expressed as a percentage of the predicted values for sex, height, and age. Predicted values were based on the formula of Knudson et al. [25] for those over the age of 10 years, and Polgar and Varuni [26] for those 6–10 years of age.

2.4. Statistical analysis

Contingency tables and Fisher's exact test were used for association studies, and relative risk was calculated. The t test was used to compare continuous data between the two groups of patients. Survival analysis was performed using the Kaplan–Meier method and assessed by the log-rank test. For all analyses SPSS statistical package was used. A p value of 0.05 or less was considered significant.

3. Results

3.1. Patient demographics and clinical features

Baseline characteristics of the pediatric CF cohort are shown in Table 2. A total of 106 patients born between 1973 and 2009 were included. Thirty-one patients were deceased. The mean age at death was 7.5 years (median: 6.5 years, range: 1.7 to 19.8 years). Regarding age at *P. aeruginosa* infection, 50% of patients presented positive cultures before the first year of life. Spirometric results were available in 50 patients. Mean FEV_1 was 70% of that predicted for sex, height, and age (range: 18 to 119%). Mean FVC was 83% (range: 32 to 126%). Demographic and clinical variables did not show significant differences when comparing MBL-Suf and MBL-Ins patients (Table 2).

Table 1
Classification of the clinical phenotype in 66 CF patients.

Clinical phenotype	Shwachman score	Pulmonary function tests
Mild	55–75	FVC > 80% FEV_1 > 70%
Moderate	35–54	FVC 60–79% FEV_1 40–69%
Severe	<35	FVC < 59% FEV_1 < 39%

Abbreviations: FVC, forced vital capacity; FEV_1, forced expiratory volume in 1 s.

Table 2
Baseline demographic and clinical characteristics of CF patients.

		Total	MBL-Suf	MBL-Ins	p
Age at diagnosis (years)		1.4 (0–12)	1.6 (0–12)	1.2 (0.02–12)	0.477 [a]
Sex (male:female)		44:62	26:31	18:31	0.355 [b]
Health Care Insurance		61/106	37/57	24/49	0.098 [b]
Pancreatic insufficiency		106/106	57/57	49/49	–
Meconium ileus		17/99	6/54	11/45	0.080 [b]
CFTR mutations	DF508/DF508	75	41	34	0.499 [b]
	DF508/other [c]	27	15	12	
	Other [c]/other [c]	4	1	3	

Ranges are indicated between brackets.
[a] p value determined by t test.
[b] p values determined by the Chi square test.
[c] Other severe mutations: 1717-1G->A, G542X, N1303K, W1282X, G551D, DI507, 3659delC.

3.2. Distribution of MBL2 gene variants in CF patients

Structural and promoter variants in the *MBL2* gene were typed in 106 CF patients with two severe mutations in the *CFTR* gene. Frequencies are described in Table 3. For the structural variants, 36.5% of the patients were A/0 and 9.3%, 0/0. For the promoter variants, 27.6% were Y/X and 2%, X/X. The distribution of *MBL2* genotypes was found in Hardy–Weinberg equilibrium for both structural (p = 0.39) and promoter (p = 0.79) variants.

3.3. Effect of the MBL2 genotype on disease severity

In 66 patients the clinical phenotype was established. Twenty-one were classified as severe and 45 as non-severe (including moderate and mild). According to the *MBL2* genotype, 39 were MBL-Suf and 27, MBL-Ins.

MBL insufficiency was significantly more frequent in the group of patients with a severe phenotype (62%) than in the group with a non-severe phenotype (31%, p = 0.03). MBL deficiency was associated with a 3.5 times higher risk of having a severe phenotype (95% CI: 1.2–10.3) (Fig. 1).

3.4. Influence of the MBL2 genotype on lung function

Measurements of FEV_1 and FVC, considered for the assignation of the clinical phenotype, were available in 50 patients, 32 MBL-Suf and 18 MBL-Ins. No differences were found in the mean values of FEV_1 and FVC between both groups (FEV_1: 71% vs 69%; FVC: 85% vs 84%, respectively).

3.5. Follow-up of lung function

In a group of 28 patients, 17 MBL-Suf and 11 MBL-Ins, FEV_1 measurements were collected at 7, 8, 9, and 10 years of age. At 7 years, lung function of MBL-Ins patients was significantly reduced compared with MBL-Suf patients (p = 0.028). FEV_1 values in MBL-Ins patients were below those of MBL-Suf patients, although these differences were not statistically significant (Fig. 2).

Table 3
Genotype frequencies of *MBL2* gene polymorphisms in CF patients.

MBL2 genotype	CF patients	
	n	%
Structural		
A/A	52	54.2
A/B	22	22.9
A/D	13	13.5
All A/0 [a]	35	36.5
B/B	6	6.3
B/D	3	3.0
All 0/0 [b]	9	9.3
Total	96	100
Promoter		
Y/Y	68	70.8
Y/X	26	27.1
X/X	2	2.1
Total	96	100
Haplotypes		
YA/YA	35	36.5
YA/Y0	26	27.1
Y0/Y0	8	8.3
YA/XA	15	15.6
XA/Y0	9	9.4
XA/XA	2	2.1
Y0/X0	1	1.0
Total	96	100

For estimation of genotypic frequencies, only the eldest sibling of each of the ten pairs of siblings was included.
[a] A/0 = A/B + A/D.
[b] 0/0 = B/D + B/D.

3.6. MBL2 genotype and age at acquisition of *P. aeruginosa*

Data on the first positive culture for *P. aeruginosa* were available in 76 patients; 43 were MBL-Suf and 33 MBL-Ins. The age at first *P. aeruginosa* infection was significantly lower in the MBL-Ins group than in the MBL-Suf group ($p = 0.035$) (Fig. 3).

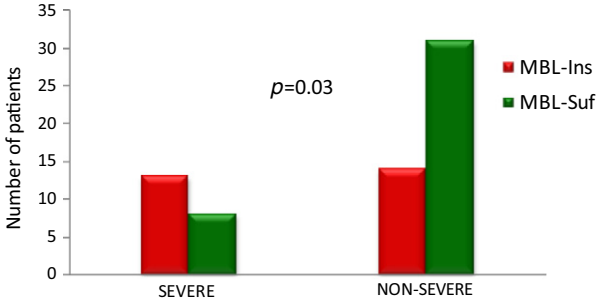

Fig. 1. Distribution of patients with severe and non-severe phenotypes in MBL-Ins and MBL-Suf groups. Among the 21 patients with a severe phenotype, 13 belonged to the MBL-Ins group (red bars) and 8 to the MBL-Suf group (green bars). Of the 45 patients with a non-severe phenotype, 14 were MBL-Ins and 31 MBL-Suf. The association study was tested using the Fisher exact test ($p = 0.03$).

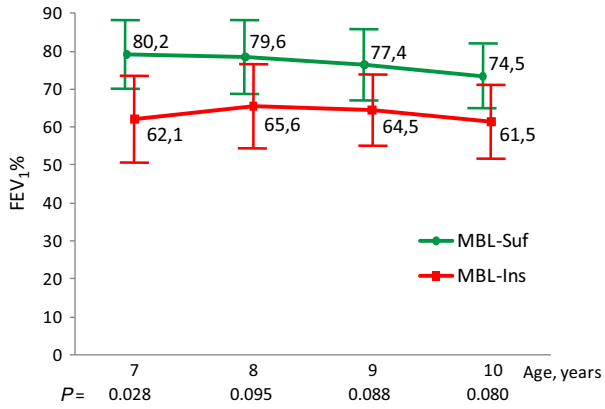

Fig. 2. Lung function in patients between 7 and 10 years old. Patients were stratified according to whether they belonged to the MBL-Suf group (n = 17, green line) or the MBL-Ins group (n = 11, red line). Mean FEV_1 in % is indicated. Error bars represent the standard errors of the mean. The individual *p* values for each year are given.

3.7. MBL2 genotype and survival

Kaplan–Meier survival curves were plotted to assess the distribution of age at death in 82 patients; 46 were MBL-Suf and 36 MBL-Ins. No statistically significant differences were found in age at death between MBL-Suf and MBL-Ins groups (log-rank test, $p = 0.482$).

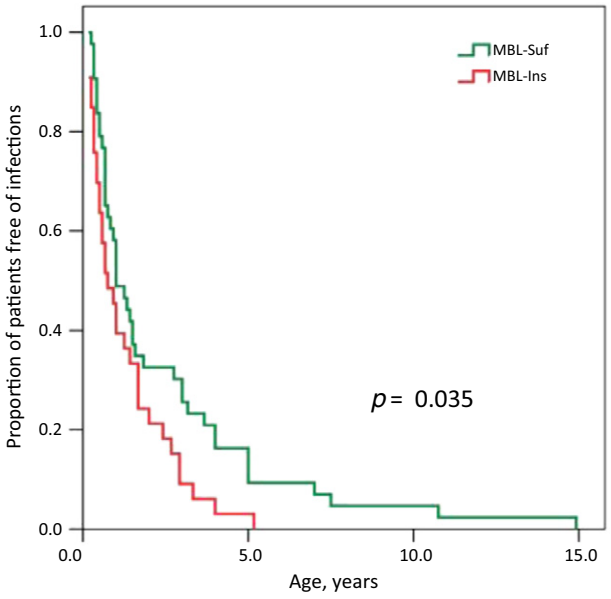

Fig. 3. Kaplan–Meier plot for time to first positive culture of *P. aeruginosa*. The proportion of patients free of infection with *P. aeruginosa* versus age is plotted for MBL-Suf (n = 43, green line) and MBL-Ins (n = 33, red line). The survival probability differences were tested using the log-rank test ($p = 0.035$).

4. Discussion

The course of the disease in CF patients is highly variable, even among those with the same *CFTR* genotype. Modifier genes as well as environmental factors are thought to contribute to this variability. In the present study, we assessed the influence of *MBL2* gene variants on disease severity, age at acquisition of *P. aeruginosa*, and survival in a series of children with CF. To exclude variables that could affect the phenotype, the following considerations were taken into account: i) patients belonged to a single medical center to reduce possible variability in the status of the disease caused by differences in treatment and measurement of clinical parameters; ii) all patients had two severe mutations to minimize the effect of the *CFTR* genotype on the disease; iii) for the analysis of association of *MBL2* gene variants with the clinical phenotype only patients between 5 and 9 years were included in order to obtain a homogeneous group with as many lung function studies as possible.

According to the effect on protein level, *MBL2* genotypes are classified into different groups. Some studies use high, intermediate, and low MBL expression groups, whereas most studies classify the genotypes into MBL-sufficient and MBL-insufficient groups [9,14,16,27]. The latter classification was used in the present study. Grouping according to genotype instead of protein levels may lead to clearer results, since genotype is a non-variable parameter [15].

Up to now, in the vast majority of studies on *MBL2* and CF, the lung phenotype was used as a marker of clinical severity [2,12,13,15–17]. In this line, a recent meta-analysis revealed a significant correlation between MBL insufficiency and reduced FEV_1 in adult cohorts [14]; however, in five pooled pediatric cohorts no significant effect of *MBL2* genotype on FEV_1 was observed [14]. In fact, Muhlebach et al. [17] suggested that the effect of MBL insufficiency on lung function may become apparent after adolescence.

In the present study, in addition to lung involvement measured by FEV_1, we describe the global clinical phenotype based on the Shwachman score and lung function studies. The Shwachman score remains a useful tool for monitoring the severity of cystic fibrosis, adequately reflecting functional impairment as well as chest radiography and tomography changes [23]. This allows us to classify patients as mild, moderate, and severe in a more objective manner. We found that patients with *MBL2* genotypes associated with low production of protein had an increased risk of presenting a severe phenotype, 3.5 times higher than those in the MBL-Suf group. Nevertheless, there was no difference in the FEV_1 value between MBL-Suf and MBL-Ins patients, probably due to the fact that most patients who lacked data on lung function had a severe phenotype.

In the group of 28 patients who had a follow-up of lung function for at least four years, FEV_1 at 7 years of age was significantly reduced in MBL-Ins patients compared with MBL-Suf patients. In the follow-up, there was a trend towards maintaining reduced lung function in the MBL-Ins group. Garred et al. [2] observed in a cohort of patients of varied ages followed for 9 years that lung function remained significantly reduced in MBL-Ins patients compared with those that were MBL-Suf. In a multicenter study of pediatric patients, Dorfman et al. [16] found that in both groups at 10 years of age FEV_1 was similar but that the rate of decline of FEV_1 was significantly steeper in the *MBL2* genotypes associated with low expression [16]. Collectively, these data suggest that the effect of *MBL2* expression could be a critical determinant of the severity of lung disease at a young age in CF patients.

In previous studies, MBL deficiency was associated with earlier acquisition of *P. aeruginosa* [14,16]. In our series, although most patients showed early ages at infection, in the analysis of time to event it was observed that MBL-Ins patients presented an earlier age at first infection of *P. aeruginosa* than those who were MBL-Suf. This modulating effect probably reflects the critical role of MBL in the first defense against bacterial colonization. In recent studies, deficiency of other proteins of the lectin pathway, such as ficolins (*FCNs*) and MBL-associated serine proteases (*MASPs*), also showed association with earlier onset of *P. aeruginosa* [19].

Survival studies have produced conflicting results, with authors who claimed a lower survival rate in MBL-Ins patients [18,28] and others who did not find such association [13,27]. Our analysis did not show statistically significant differences in age at death between MBL-Suf and MBL-Ins patients.

Up to our knowledge, there are very few studies on CF modifying factors in Latin-American countries where socioeconomic conditions of patients seen at public hospitals are far from ideal. One of them, by Faria et al. in a Brazilian CF population, did not find association between *MBL2* polymorphisms and lung disease severity. However, the authors did not specify how patients were grouped according to *MBL2* genotype. In our series, we observed the influence of *MBL2* deficiency on the disease course, similarly as described in developed countries of North America and Europe [2,11,12,15–19]. Our study highlights the role of the *MBL2* gene as a modulating factor in CF, determining the course of the disease in addition to environmental factors.

Finally, in this group of CF patients, MBL insufficiency was associated with detrimental progression of the disease. In addition to the contribution to a better understanding of the pathogenesis of CF, the recognition of modifiers such as the *MBL2* gene paves the way for the development of new therapeutic strategies. Although MBL replacement therapy appears to be effective in restoring the lectin pathway of complement in adults and children with low MBL, there is still no evidence from randomized clinical trials that show a beneficial effect in the treatment or prevention of infections [14]. Moreover, genetic testing of modifying factors such as MBL may lead to early identification of at-risk patients, so they can be closely monitored by their physicians [16] and receive more intensive medical care.

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