

## Cardiovascular risk and mannose binding lectin in patients with rheumatoid arthritis from southern Brazil

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### ABSTRACT

**Background:** Mannose binding lectin (MBL) appears to be involved in susceptibility to rheumatoid arthritis (RA), in the inflammatory process and in the genesis of atherosclerotic disease.

**Objective:** To study the association of MBL serum levels and its genotypic variation with carotid arteries intimal thickness (IMT) in RA patients from Southern Brazil.

**Methods:** MBL serum levels, *MBL2* genotyping and IMT were investigated in 90 RA patients along with their demographic, clinical and laboratory profile. MBL levels and *MBL2* genotyping were evaluated in 90 healthy controls.

**Results:** A significant lower MBL serum concentration was observed in patients with RA in relation to controls (528 ng/mL vs 937.5 ng/mL,  $p = 0.05$ , respectively). The median IMT in RA patients was 0.59 mm (0.51 to 0.85 mm). There was no correlation between levels of MBL with disease activity, erythrocyte sedimentation rate, autoantibodies presence or IMT ( $p = NS$ ). A weak and negative correlation was found between MBL and CRP levels ( $Rho = -0.24$ ;  $p = 0.02$ ;). The *MBL2* variant at codon 54 (variant B) and HYP A haplotype were the most frequently observed in the RA sample (67.5% and 31.7%). *MBL2* wild type (A/A) were associated with lower IMT when compared with heterozygotes (A/O;  $p = 0.04$ ) and low producers (O/O;  $p = 0.05$ ). In addition, high producers genotypes had lower levels of CRP when compared with medium ( $p = 0.04$ ) or with low producers ( $p = 0.05$ ).

**Conclusion:** RA patients had lower MBL levels than controls. MBL were negatively associated with CRP serum levels; low MBL genotypes producers increased thickness of the IMT than high producers.

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### 1. Introduction

Patients with rheumatoid arthritis (RA) have life expectancy of 5 to 10 times lower than the general population due to increased cardiovascular risk, which is considered to be by 2 to 3 times higher [1]. The reason why these patients have early and accelerated atherosclerosis has been the subject of several studies [1]. Recently, it has been accepted that the chronic inflammatory process seen in RA is related to the genesis and development of atherosclerotic plaques [1, 2].

The mannose binding lectin (MBL) is a serum protein produced in the liver, belonging to the collectin family. Its main function is to activate the complement system via the lectin pathway [3–6]. MBL production is regulated by the *MBL* gene (*MBL2*), that is located on chromosome 10, and consists of 4 exons and 3 introns [6–8]. Mutations in exon 1 of the *MBL2* gene and variations in the promoter region are known to affect MBL serum levels [6]. The prevalence of mutations in the exon 1

varies according to the population's ethnic background: variations of codon 57 are common in Africans, rare in Europeans and absent in Eskimos. Yet, mutation at codon 54 is a rare finding in Africans but quite common in Europeans and Chinese [3, 9, 10].

The serum concentrations of MBL fluctuate between 0 and 5000 ng/mL in healthy persons [6]. Subjects with levels under 100 ng/mL are considered to have low levels (or MBL deficient); those with levels between 100 and 1000 ng/mL are said to have medium levels and those above 1000 ng/mL, to have high levels [8].

The influence of MBL in the development and prognosis of cardiovascular disease is complex and not fully understood. There are paradoxical observations. Some authors have shown that MBL has protective role against the development of atherosclerosis through the clearance of apoptotic cells and cell debris and against infections such as *Chlamydia pneumoniae* and *Helicobacter pylori* [11–13]. However, other studies reported that high levels of MBL may lead to excessive activation of the complement via the lectin pathway, resulting in a pro-atherogenic inflammation and stimulating atherosclerosis or ischemic heart disease [14]. This uncertainty in the understanding of MBL function is directly

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related to the lack of a deeper understanding of the role of this protein in immunity and inflammation [10, 12].

In the context of RA, there are several studies with conflicting results relating serum levels of MBL and its genetic polymorphism with susceptibility, severity, radiographic progression and disease activity [3, 5, 8, 15–18]. Reports on the role of MBL in RA involved in atherogenesis are also diverse. Troelsen et al. reported a dual role for MBL in the cardiovascular risk of RA patients, with high MBL levels having increased risk for myocardial ischemic disease [14]. On other hand in a later study, these same authors, after analysis with linear and quadratic models of the measurement of carotid media intima thickness (IMT) in relation to MBL, found a correlation between low levels of MBL and IMT [19].

In the present study, we aimed to investigate the association between MBL and cardiovascular risk through the carotid artery IMT in patients with RA from a Southern Brazilian population. This association was studied through MBL serum levels and MBL2 genotyping.

## 2. Materials and methods

### 2.1. Patients and ethical issues

The study included 90 Southern Brazilians patients with RA, accompanied in a single Rheumatology Clinic from a University Hospital during the period of February 2015 to February 2016. All patients met the classification criteria for RA according to the 1987 American College of Rheumatology (ACR) or ACR/EULAR (European League Against Rheumatism) Classification criteria [20]. This study was approved by the local Ethics in Local Research Committee and all included patients signed consent. For control purposes the MBL in 90 healthy volunteers belonging to the hospital staff, who had no chronic rheumatic disease and no relatives with RA was measured.

### 2.2. Clinical and laboratory data

Demographic, clinical and laboratory data were obtained through interviews with the patient and/or obtained from the medical records. Collected data included age, gender, ethnicity, smoking, presence of diabetes mellitus (DM), hypertension (HBP), dyslipidemia and statin use and body mass index (BMI). We also analyzed items considered as potential atherosclerotic risk factors associated with AR: presence of anti-citrullinated peptide (anti-CCP), rheumatoid factor (RF), age at disease onset, duration of disease, erythrocyte sedimentation rate (ESR), C-reactive protein (CRP), functional index of Steinbrocker [21] and disease activity index calculated by the DAS 28- ESR (Disease Activity Score 28 - ESR) [22].

### 2.3. Measurement of MBL serum levels and genotyping of MBL2 gene

The serum levels of MBL were determined by the ELISA method (Enzyme Linked Immuno Sorbent Assay) utilizing the anti-MBL monoclonal antibody HYB 131-01 (BioPorto Diagnostics A/S, Copenhagen, Denmark). Individuals with serum MBL < 100 ng/mL were considered low producers or deficient; levels of 100–1000 ng/mL medium producers and levels > 1000 ng/mL high producers [8].

Genotyping was performed by PCR amplification of the promoter region (position –550, –221 and + 4, representing the loci H/L, X/Y and P/Q respectively) and of exon 1 (codons 54, 57 and 52) of the MBL2 gene and subsequently sequenced using suitable primers, in according to Goeldner et al. [8] Exon 1 mutations comprise exchange of nitrogenous bases in codons 54, 57 and 52, and are respectively referred to as variant B (GGC to GAC, substituting glycine for aspartic acid), variant C (GAA GGA by substituting glycine for glutamic acid) and variant D (CGT by TGT, replacing cysteine by arginine). The wild-type allele is called A [3, 4, 6, 7, 15, 23]. The homozygous for the mutation O/O (where O can be B, C or D) were considered to be low MBL producers; the heterozygotes individuals (A/O) are considered to be intermediary MBL

producers and those with homozygous wild allele (A/A) are considered to be high MBL producers.

Polymorphic variations in the promoter region were expressed by the haplotypes HYP, LYQ, LYP, LXP [6]. Low MBL serum concentrations are associated with haplotype LXP [6, 15].

### 2.4. Measurement of IMT

The measurement of IMT was performed by a single investigator, blind for clinical data, with Esaote® ultrasound apparatus, high resolution, model MyLab40, in B-mode and with a linear transducer of 18 MHz. The patients were studied in a quiet, air-conditioned environment at 22 °C, in the supine position with the neck extended and rotated 45° contralateral to the examined side. The carotid artery was observed in transverse and longitudinal planes, with measurement carried out at a distance of 10 to 20 mm of the carotid bifurcation, in the distal vessel wall [24]. The examination was performed on both sides; for statistical purposes the highest value was considered. The reference values used were 0.4 to 0.7 mm as normal IMT; 0.8 to 1.4 mm as thickened IMT (subclinical atherosclerosis); values greater than or equal to 1.5 mm, as atheroma [25].

### 2.5. Statistical analysis

Data were collected in frequency and contingency tables. Measures of central tendency were expressed as mean and standard deviation (SD) for parametric samples and median and interquartile range (IQR) for non-parametric samples. Normality was judged by the Kolmogorov Smirnov test.

Comparison between two numerical samples were made using Mann Whitney test when the sample distribution was nonparametric and unpaired *t*-test when parametric. The comparison of three samples was performed by Kruskal Wallis test (nonparametric) and one way Anova (Parametric). Nominal data were compared by Fisher's and chi-square test. Correlation studies were done using Spearman test. When a variable was associated/correlated with several others, independence was tested by multivariate regression. The adopted significance was of 5% and the calculations were made with the aid of the software Medcalc 14.0.

Direct counting was used to estimate the genotypes, haplotypes and allele frequency. Deviations from Hardy-Weinberg equilibrium and the assumption of homogeneity between the distributions of haplotypes were tested using Arlequin 3.1 software.

## 3. Results

### 3.1. Description of studied sample

The descriptive data of the RA sample are on Table 1. In the control group there was 69/90 females (76.6%) with age from 20 to 81 years of age (median 52 years; IQR = 45.0–61.0). Pairing date showed *p* = 0.33 for gender and 0.10 for age.

In the RA patients the median carotid artery IMT was 0.59 mm (0.51 to 0.85 mm), with 30% (27/90) of them having IMT higher than 0.8 mm, characterizing subclinical atherosclerosis. MBL serum levels in RA and controls are on Fig. 1.

Concerning MBL2 genetic analysis, the exon 1 variations found in this RA sample were: 67.5% variant B, 10% variant C and 22.2% variant D. The polymorphic variations of promoter regions were: 19.6% LXP; 37.8% HYP; 16.2% LYQ; 26.4% LYP. The haplotype HYP A was the most frequent and found in 31.7% of the patients. We considered A/A as high MBL producers (found in 32.4%); A/O as medium MBL producers (found in 40.25%) and O/O as low MBL producers (found in 27.7%).

**Table 1**  
Demographic, clinical, serological and treatment data in 90 rheumatoid arthritis patients.

Gender	17.7% (16/90) males/82.2% (74/90) females
Auto declared ethnic background	Afrodescendants: 28.8% (26/90) Europeans: 71.1% (64/90) Asian: 1.1% (1/90)
Age (years)	33–82; median 59 (48–65)
Disease duration (years)	5–35; median 13 (10–18.25)
Positive anti-CCP	83.3% (75/90)
Positive rheumatoid factor	76.4% (69/90)
DAS28	0.42–8.07; median 3.26 (2.67–4.37)
Erythrocyte sedimentation rate (mm)	1–106; mean 41.68 ± 24.93
C reactive protein (mg/dL)	0.1–80; mean 15.9 (8.75–24)
Total cholesterol (mg/dL)	113–371; mean 182.5 ± 39.17
Triglycerides (mg/dL)	42–313; media 130.4 ± 54.95
HDL cholesterol (mg/dL)	46–126; median 50 (41.75–60.25)
LDL cholesterol (mg/dL)	46–293; media 103.8 ± 35.09
Body mass index (BMI; Kg/m <sup>2</sup> )	15.79–37.65; media 27.08 ± 4.75 Low BMI (BMI < 18.5) = 3/19 (3.3%) Normal (BMI 18.5–24.9) = 31/90 (34.4%) Overweight (BMI 25–29.9) = 31/90 (34.4%) Obese (BMI > 30) = 25/90 (27.7%)
Steinbrocker functional class	Class 1–43/90 (47.8%) Class 2–36/90 (40%) Class 3–9/90 (10%) Class 4–2/90 (2.2%)
Exposure to tobacco	30/90 (33.3%)
Age at disease onset (years)	22–66; median 45 (IQR = 29–50)
Cerebral vascular accident/ myocardial infarction	0/90
Diabetes mellitus	14/90 (15.5%)
Arterial hypertension	38/90 (42.2%)
Dyslipidemia	38/90 (42.2%)
Methotrexate users	56/90 (62.2%)
Glucocorticoid users	39/90 (65.0%)
Leflunomide users	49/90 (81.6%)
Use of biologic drugs	33/90 (36.6%)

**3.2. Carotid IMT according to RA variables**

None of the variables as gender, ethnic background, tobacco exposure, autoantibodies (RF and anti CCP), presence of rheumatoid nodules, DAS-28, ESR and CRP, cholesterol levels, triglycerides, HDL and LDL cholesterol levels showed association/correlation with IMT (all *P* = NS).

IMT was significantly higher in patients with hypertension (*p* < 0.0001), dyslipidemia (*p* < 0.0001), high BMI (*p* = 0.04) and using biologic drugs (*p* = 0.002). Methotrexate use showed a trend towards a lower IMT value (*p* = 0.07). In addition, a positive correlation was found between IMT and patients' age (Rho = 0.51; 95%CI = 0.34–

0.65; *p* < 0.0001) and age at disease onset (Rho = 0.43; 95%CI = 0.24–0.59; *p* < 0.0001). Analysis through logistic regression of all variables with *p* < 0.1, IMT was found associated with hypertension and age at disease onset as independent variables.

**3.3. IMT, MBL serum levels and MBL2 genotypes**

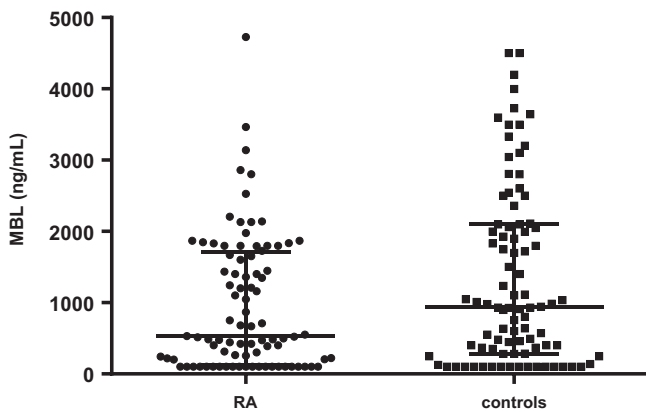
MBL serum levels were not related with demographic, clinical or serological RA profile (all with *p* = NS). A negative but very weak correlation was found between CRP and MBL levels (Rho = −0.24; 95%CI = −0.43 to −0.02; *p* = 0.02). Correlation of MBL serum levels and IMT was negative (*p* = 0.38) even when correct for CRP levels (*p* = 0.08). Comparison of IMT of the patients classified as MBL deficient (median = 0.68 mm; IQR = 0.49–0.93 mm) with the group of low producers (median = 0.68 mm; IQR = 0.48–0.85 mm) and high producers (median 0.57 mm; IQR = 0.51–0.85 mm) showed no difference (*p* = 0.34). Patients with IMT < 0.8 mm had a medium serum MBL level of 486 ng/dL (IQR = 100–1800) and those with IMT ≥ 0.8 mm of 524 ng/dL (IQR = 100–1668) with *p* = 0.79. Association between MBL serum levels and MBL2 polymorphisms was found, as expected. Fig. 2.

When the analysis of MBL genotypes was done, no associations could be established with clinical, serological and demographic variables (all with *p* = NS). However, it was found that recessive patients (low producers) had higher levels of serum CRP (Fig. 3-A) and higher carotid IMT (Fig. 3-B) but the DAS-28 ESR was similar in the three groups (Fig. 3-C).

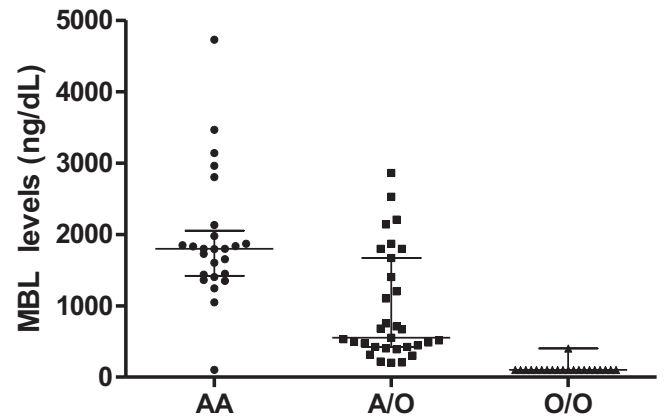
**4. Discussion**

Despite the fact that RA patients have increased risk of atherosclerosis, we could not associate presently any of RA clinical variables with increased IMT to explain why this happens. Our RA population had mean disease duration of >10 years, time enough to allow possible associations to appear. The independent associations found here were those already known as classical risk factors for atherosclerosis such as hypertension, age and dyslipidemia. An important consideration is that the patients were all followed at a University Hospital, where disease activity is treated aggressively, use of glucocorticoid is minimized and the conventional risk factor for atherosclerosis is treated. Nevertheless our RA patients had significantly lower levels of MBL than controls. If MBL levels would impact the atherosclerosis risk, this could be an explanation why RA patients are prone to this complication.

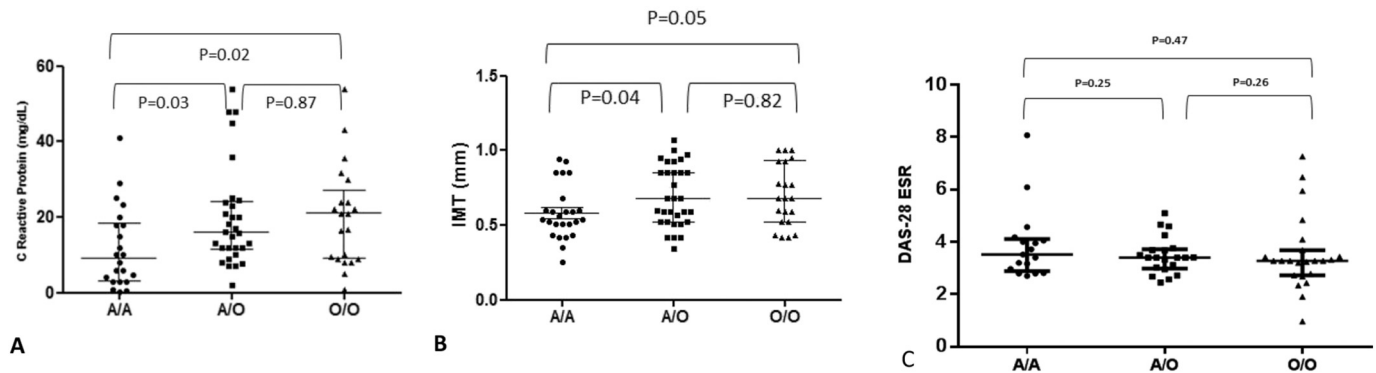
Some authors believe that MBL levels are important in the appearance of RA itself. Ipe et al. [3], studying Chinese RA patients, found



**Fig. 1.** Comparison of mbl (mannose binding lectin) serum levels in ra patients and controls RA patients with values from 100 to 4725 ng/mL (median of 528 ng/dL) and controls with from 100 to 4500 ng/dL (median 937.5 ng/dL) with *p* = 0.05.



**Fig. 2.** Mannose binding lectin (mbl) serum levels according to mbl2 genotypes Serum levels in AA individuals (median levels 1800 ng/dL; IQR = 1417–2054); A/O (median level 550 ng/dL; IQR = 202–2862); and O/O (median levels 100 ng/dL; IQR = 100–199) with *p* < 0.0001.



**Fig. 3.** MBL (mannose binding lectin) 2 genotypes, c reactive protein (CRP) and carotid intima media thickness (IMT) in rheumatoid arthritis patients. A - Graphic showing values of CRP in A/A with median value of 9.0 mg/dL (IQR = 6.0–20.8 mg/dL), A/O with median value of 15.9 mg/dL (IQR = 12.0–24.3 mg/dL) and O/O with median value of 21.0 mg/dL (IQR = 13.2–29.1 mg/dL) genes for MBL production. B - Graphic showing IMT in patients with A/A for MBL with median values of 0.54 mm (IQR = 0.47–0.64); in heterozygous with median values of 0.68 mm (IQR = 0.52–0.85) and O/O with median values of 0.68 mm (IQR = 0.52–0.93). C - Graphic showing DAS (Disease activity score)-28 ESR (erythrocyte sedimentation rate) values in A/A with median value of 3.56 (IQR = 2.89–4.11), A/O with median values of 3.40 (IQR = 2.99–3.72) and O/O with median values 3.28 (IQR = 2.73–3.69) genes for MBL production.

lower levels of MBL in their sample and concluded that MBL deficiency predisposes to RA development. Graudal et al. [17] also found low levels in a Danish RA population and stated that MBL insufficiency may be a contributing pathogenetic factor to this disease. Contrary to that, Saevarsdottir et al. [16], found higher MBL levels in a cohort of Iceland RA patients. Goeldner et al. [8] suggested that the levels of MBL has no central role in the development of RA, but it is a likely cofactor in its genesis. Conflicting reports on MBL and the risk of RA may, at least partly, be due to ethnic differences in the studied patient cohorts. Lipscombe et al. [10], Madsen et al. [9] and Ip et al. [3] found that the MBL2 B variant in exon 1 is the most common in European and Chinese but quite rare in Afrodescendants, in which variant C is the commonest. In our cohort variant B was the most common, corroborating the predominance of Eurodescendants in the casuistic.

In the present study, serum levels of MBL did not associate with disease activity or severity markers. Our results are consistent with those of Graudal et al. and Geijin et al., endorsing the hypothesis that MBL is not a good marker in this context [3, 5, 15]. However, interesting findings about the influence of MBL on IMT were observed. Firstly, MBL2 genotypes, but not serum levels of MBL, were linked to atherosclerosis evaluated by IMT. MBL serum levels although genetically determinate may also suffer influences of punctual factors such as infections, for example. Genotypes reflect how MBL levels behave most of the time and it is a more constant variable. The second interesting observation is that MBL genotypes show an inverse relationship of MBL production, not only with IMT but also with serum CRP levels, already recognized to be linked to the atherosclerotic process [26]. Lower MBL producers had higher IMT and higher CRP serum levels. This clearly points out to a beneficial role of this protein in the atherosclerotic process. Our results agree with the second study of Troelsen et al. [19], which correlated higher levels of MBL in RA patients with lower carotid IMT.

The molecular mechanisms by which MBL may exert its protective effects in atherosclerosis have not yet been established. Beyond its role in the activation of complement, MBL is also a member of the defense collagen family of proteins, which includes the class A scavenger receptors. [27] It has been shown that defense collagens are capable of rapidly boosting phagocytic activity when bound to the particle to be ingested. So MBL is an efficient contributor to removal of apoptotic cells, cellular debris and modified lipoproteins. According to Fraser et al., [27] MBL also reduced significantly the levels of free cholesterol accumulation in monocytes and human monocyte-derived macrophages that ingested oxidized LDL, while enhancing high-density lipoprotein-specific cholesterol efflux from these cells.

Concluding, MBL serum levels were lower in RA patients in relation to healthy controls from Southern Brazil. This marker cannot be used to determinate disease activity nor to discriminate any clinical RA pattern.

However, RA patients that are MBL low producers presented higher carotid IMT, suggesting a protective role for MBL2 in the atherogenic process.

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#### Disclosures

All authors have no conflict of interest.

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