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Leukocytes and complement in atherosclerosis

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b. Mannose binding lectin 2 haplotypes do not affect the progression of coronary atherosclerosis in men with proven coronary artery disease treated with pravastatin

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ABSTRACT

Introduction: Mannose binding lectin (MBL) is one of the three initiators of complement activation. Polymorphisms of the MBL2 gene and its promoter, and especially haplotypes, determine MBL plasma levels. MBL deficiency has been associated with the development of atherosclerosis. We evaluated whether the rate of angiographic progression of coronary atherosclerosis during pravastatin treatment was associated with MBL2 haplotypes in REGRESS, a placebo-controlled 2 years intervention study.

Methods: Three polymorphic sites in exon 1 (rs1800450, rs1800451, and rs5030737) of the MBL2 gene and 2 sites (rs7096206, and rs11003125) in the promoter region were genotyped in 398 subjects. Genotyping was performed using Applied Biosystems® TaqMan® Genotyping Assays. We divided the group in high, intermediate and low MBL2 secretor haplotypes. Quantitative coronary angiography was performed. Endpoints were mean segment diameter (MSD) and minimum obstruction diameter (MOD) established by quantitative coronary angiography.

Results: At inclusion, 50.1, 31.7 and 17.6% of the patients in the REGRESS cohort carried the high, intermediate and low MBL2 secretor haplotypes, respectively. In 0.6% of the patients, the haplotype was not informative. There were no baseline differences between the MBL2 haplotypes for age, BMI, lipid levels, leukocyte counts, CRP, MSD and MOD. The intermediate MBL2 placebo group showed the greatest increase in MSD compared to the low MBL2 group ($P=0.03$). No difference was found for the change in MOD. No significant interaction between MBL2 haplotype groups and pravastatin therapy was observed.

Conclusions: In men with proven coronary artery disease, MBL2 secretor haplotypes are not associated to the rate of progression of coronary sclerosis nor does pravastatin treatment influence progression based on MBL2 haplotypes.

INTRODUCTION

Inflammation is closely associated to atherosclerosis (1-2). In the past few years several pro-inflammatory genes have been identified, which have been suggested to play a role in atherosclerosis. One of these genes is the Mannose Binding Lectin (MBL) gene. MBL deficiency has been associated to coronary artery disease (CAD), increased intima-media thickness in carotid arteries (3-5) and atherosclerosis in different clinical situations (6-8). Recent work from our laboratory shows that MBL deficiency may lead to a disturbed metabolism of postprandial VLDL1 lipoproteins (9), potentially favoring atherosclerosis.

MBL is an important activating factor of the lectin pathway of the complement system (10,11). The MBL2 gene codes for the active MBL protein and has three known mutations: allele B at codon 54 (G54D, rs1800450), allele C at codon 57 (G57E, rs1800450) and allele D at codon 52 (R52C, rs5030737) (11,12). These mutations lead to structural abnormalities and cause MBL deficiency (11). The wild-type codon is allele A. Moreover, there are two mutations in the promoter region at -550 (H/L, rs11003125) and -221 (Y/X, rs7096206), which result in a decreased synthesis of the protein (12-14). The alleles interact with each other to form MBL2 'secretor haplotypes' producing high, intermediate and low MBL levels (12,13).

The role of MBL in atherosclerosis is unsettled since several recent studies have suggested that not only low, but also high MBL levels are associated with atherosclerosis (15-19).

Statins have been shown to reduce the atherosclerotic burden in different groups of patients (20-22) and are able to modulate complement components in normolipidemic subjects with CAD (23). Currently, no data are available on the role of MBL in the progression of coronary atherosclerosis in prospective intervention studies with statins. In this study, we aimed to investigate the relation between the MBL2 secretor haplotypes and the progression of coronary atherosclerosis in patients treated with pravastatin compared to placebo in the REGRESS (Regression Growth Evaluation Statin Study) population (20).

MATERIALS AND METHODS

Subjects

Samples collected in the REGRESS cohort were used (20). The REGRESS protocol has been described in detail elsewhere (20). In brief, REGRESS was designed as a double-blind, placebo-controlled, multicenter study to assess the effect of pravastatin treatment on the progression and regression of coronary atherosclerosis. All patients were men of Caucasian descent; they were <70 years of age, and had angiographically documented CAD (>50% stenosis of 1 major vessel). Patients who had unstable angina or who suffered a myocardial infarction within the preceding 6 months of the study were excluded; angina pectoris classification was based on

the Rose questionnaire. All patients had total cholesterol levels between 4 and 8 mmol/L and TG levels <4 mmol/L.

The present analysis was approved by the Local Ethical Committee of the Sint Franciscus Gasthuis and the Medical Ethical committee of the Leiden University Medical Center.

Analytical methods

All lipid laboratory tests were carried out at the Lipid Reference Laboratory (Atlanta, Ga). Serum lipids, blood cell counts and high sensitive CRP (hsCRP) were measured in fasting blood samples by standard techniques and LDL was calculated according to the Friedewald formula (20).

Quantitative Coronary Arteriography

The quantitative coronary arteriographic procedures have been described in detail elsewhere and include the mean segment diameter (MSD) and minimum obstruction diameter (MOD) on a per patient basis (20).

DNA analysis and assignment of haplotype status

Genomic DNA was isolated from white cells via standard procedures, dissolved in 10 mmol/L Tris, 1 mmol/L EDTA, pH 8.0, and stored at 4°C. 3 polymorphic sites in exon 1 (rs1800450, rs1800451, and rs5030737) of the MBL2 gene and 2 sites (rs7096206, and rs11003125) in the promoter region were genotyped in 398 subjects. Genomic DNA samples were genotyped using TaqMan® SNP Genotyping Assays specific for each one of the polymorphic sites and according to the manufacturer's instructions. The assays were performed on a GeneAmp PCR system 9700 with TaqMan® master mix (Applied Biosystems®) and allelic discrimination was done on a 7900HT fast real-time PCR system (Applied Biosystems®). To ensure consistency between runs, samples of known genotypes were repeated in every analysis.

Patients were considered to have the high MBL2 secretor haplotypes if they had the HYA and LYA haplotypes. The haplotypes LXA, HYD, HYBD and HXA were characterised as the intermediate MBL2 secretors and the haplotypes LYB and LYC as the low MBL2 secretors (12,13).

Statistics

We first checked whether the genotype distributions of the five MBL2-gene SNPs were in Hardy-Weinberg equilibrium using one-degree of freedom Chi-square tests. Subsequently, baseline patient characteristics were compared between genotypes using ANOVA, Chi-square tests, or Kruskal-Wallis tests where appropriate. MSD and MOD changes during the trial were compared between genotypes using ANOVA with baseline values as covariates, and the occurrence of

cardiac events during the trial were analysed with the log rank test. Patients with distinctive haplotypes in the low (LYC) and intermediate (HYD, HYBD, HXA) MBL2 groups represented only a small portion of the total group. Therefore, the statistical analysis was carried out for the MBL2 secretor haplotypes, rather than the distinctive haplotypes in the three groups. We estimated individual haplotypes and the association between haplotypes and patient characteristics using Phase (24) and the algorithms developed by Souverein et al (25). Concerning power analysis, the current sample size provided at least 80% power to detect a significant association between MBL2 genotypes and MSD/MOD change if the mean difference is larger than about 0.3 standard deviations. This was the case for rs7096206, rs11003125 and rs1800450. Because the minor allele frequencies were lower, the mean difference should be larger than 0.46 for rs5030737 and larger than 0.76 for rs1800451. The standardized mean difference (or Cohen's effect size) is considered moderate between 0.2 and 0.5 and large > 0.5 .

RESULTS

MBL2 distribution and baseline characteristics (Tables 1-3)

The number of REGRESS patients of whom DNA was available and with at least one genotyped MBL2 SNP was 398. The relative frequency of the SNPs is listed in Table 1. Only the rs7096206 and rs1800450 SNPs were not in Hardy-Weinberg equilibrium (Table 1). The distribution of the genotypes was similar with those reported in other studies (12,13).

The MBL2 haplotype distribution was also comparable to other studies (12,13) (Table 2). The haplotype HXD with a relative frequency of only $0.6 \pm 0.4\%$ could not be divided in a category corresponding to one of the three MBL2 secretor levels. Therefore, we did not include the HXD haplotype in our further analysis. There were no differences between the different SNPs and haplotypes for baseline cardiovascular risk factors and the progression of CAD (Table 3).

Table 1. MBL2 allele frequencies in the REGRESS cohort

<i>Promotor region polymorphism</i>		<i>Frequency % (no)</i>	<i>HWE</i>
rs7096206	X	22.1 (156)	0.01*
	Y	77.9 (554)	
rs11003125	H	37.2 (270)	0.51
	L	62.8 (448)	
<i>Structurally encoding polymorphism</i>			
rs1800450	B	15.2 (111)	0.00*
	W	84.8 (448)	
rs1800451	C	3.1 (21)	0.55
	W	96.9 (655)	
rs5030737	D	9.6 (62)	0.18
	W	90.4 (584)	

HWE, Hardy-Weinberg equilibrium. * $P < 0.05$.

Table 2. Distribution according to extended haplotypes, and to high, intermediate and low MBL2 secretor haplotypes

Corresponding MBL	rs7096206 -221	rs11003125 -550	rs1800450 +54	rs1800451 +57	rs5030737 +52	haplotype	Rel. freq. (SEM)
High	Y	H	A	A	A	HYA	27.3 (1.9)
	Y	L	A	A	A	LYA	22.8 (1.9)
Intermediate	X	L	A	A	A	LXA	22.8 (1.9)
	Y	H	A	A	D	HYD	6.5 (1.2)
	Y	H	B	A	D	HYBD	1.8 (0.7)
	X	H	A	A	A	HXA	0.6 (0.4)
Low	Y	L	B	A	A	LYB	15.2 (1.7)
	Y	L	A	C	A	LYC	2.4 (0.7)
Undetermined	X	H	A	A	D	HXD	0.6 (0.4)

Table 3. Baseline characteristics according to extended MBL-2 haplotypes

	HYA	LYA	LXA	HYD	HYBD	HXA	LYB	LYC	P-value
Age (yrs)	55.7 (0.5)	56.8 (0.5)	56.4 (0.7)	55.1 (1.2)	59.7 (2.1)	62.4 (2.5)	55.2 (0.8)	58.4 (1.5)	0.15
BMI (kg/m ²)	59.9 (0.2)	26.1 (0.2)	25.8 (0.2)	26.3 (0.4)	26.3 (0.8)	24.9 (1.2)	26.2 (0.3)	25.2 (0.5)	0.7
LDL (mmol/L)	4.23 (0.05)	4.26 (0.06)	4.28 (0.07)	4.33 (0.11)	4.10 (0.17)	4.36 (0.32)	4.28 (0.08)	3.98 (0.16)	0.82
HDL (mmol/L)	0.94 (0.01)	0.94 (0.02)	0.95 (0.02)	0.93 (0.03)	0.93 (0.05)	1.02 (0.1)	0.93 (0.02)	0.95 (0.05)	0.99
TG (mmol/L)	1.73 (0.05)	1.77 (0.05)	1.82 (0.06)	1.86 (0.11)	1.74 (0.25)	1.35 (0.10)	1.78 (0.08)	1.78 (0.18)	0.85
CRP (mg/L)	0.90 (0.09)	0.98 (0.10)	0.98 (0.12)	0.82 (0.21)	1.62 (0.7)	ND	1.02 (0.17)	0.90 (0.34)	0.89
WBC count (*10 ⁹ /L)	7.12 (0.13)	7.15 (0.14)	7.01 (0.16)	7.07 (0.24)	7.74 (0.61)	6.85 (0.67)	7.30 (0.21)	7.80 (0.67)	0.73
Family history for CVD (%)	51	46	44	51	30	50	49	52	0.84
History of myocardial infarction (%)	47	47	48	49	70	75	48	38	0.77
Multivessel disease (%)	56.2	57.3	59.4	60.8	50.0	50.0	64.1	61.9	0.92
MSD (millimeters)	2.74 (0.02)	2.72 (0.03)	2.76 (0.03)	2.79 (0.06)	2.76 (0.11)	2.58 (0.19)	2.69 (0.04)	2.85 (0.07)	0.55
MOD (millimeters)	1.79 (0.02)	1.77 (0.02)	1.78 (0.03)	1.83 (0.05)	1.81 (0.11)	1.70 (0.22)	1.77 (0.04)	1.80 (0.06)	0.97

Data are mean±SEM. WBC count; white blood cell count. P-value by ANOVA.

Baseline characteristics of the REGRESS patients population divided into different MBL2 secretor haplotypes are listed in Table 4 and did not show any differences between the groups. There were also no differences in the use of medication between the three groups (data not shown).

Table 4. Baseline characteristics according to high, intermediate and low MBL2 secretor haplotypes

	High	Intermediate	Low	P-value
Age (yrs)	56.2 (0.36)	56.3 (0.56)	55.7 (0.73)	0.77
BMI (kg/m ²)	26.0 (0.1)	25.9 (0.2)	26.0 (0.3)	1.0
LDL (mmol/L)	4.24 (0.04)	4.28 (0.05)	4.23 (0.07)	0.78
HDL (mmol/L)	0.94 (0.01)	0.95 (0.01)	0.93 (0.02)	0.82
TG (mmol/L)	1.75 (0.04)	1.82 (0.05)	1.78 (0.07)	0.54
CRP (mg/L)	0.94 (0.07)	0.96 (0.10)	0.99 (0.15)	0.94
WBC count (*10 ⁹ /L)	7.14 (0.10)	7.06 (0.13)	7.39 (0.21)	0.35
Family history CVD (%)	49	45	50	0.64
History of myocardial infarction (%)	47	50	46	0.71
Multivessel disease (%)	56.70	59.10	63.70	0.37
MSD (millimeters)	2.73 (0.02)	2.76 (0.03)	2.71 (0.03)	0.45
MOD (millimeters)	1.78 (0.02)	1.79 (0.02)	1.77 (0.03)	0.89

Data are mean±SEM. WBC count; white blood cell count. P-value by ANOVA.

Change of MSD and MOD after 2 years of therapy (Figure 1)

Of the total group 194 and 204 patients received placebo or pravastatin, respectively. After 2 years of treatment, there were no significant differences for the occurrence of cardiovascular events between the low, intermediate and high MBL2 secretor haplotypes in the placebo (10.2% vs. 21.6% vs. 19.2% respectively, $P=0.17$) and pravastatin (6.2% vs. 6.5% vs. 7.2% respectively, $P=0.96$) treated groups.

In the placebo group, the subjects with the intermediate MBL2 secretor haplotypes showed the highest increase of MSD (0.142 ± 0.025 mm) compared to the lowest MBL2 group (0.037 ± 0.040 mm) ($P=0.03$ by ANOVA, Figure 1A). Furthermore, the intermediate MBL2 group showed a trend to a higher increase of MOD (0.154 ± 0.026 mm) compared to the low (0.113 ± 0.056) ($P=0.08$ by ANOVA; Figure 1B). In the pravastatin treated groups these differences were absent for both MSD and MOD.

Subsequently, we compared the effects of placebo and pravastatin treatments on the 2 years change of MSD and MOD. Here, the test for interaction between the different MBL2 secretor haplotypes and therapy in both placebo and pravastatin groups showed that there was no relation between the change in MSD ($P=0.16$) and MOD ($P=0.20$) and MBL2 secretor groups.

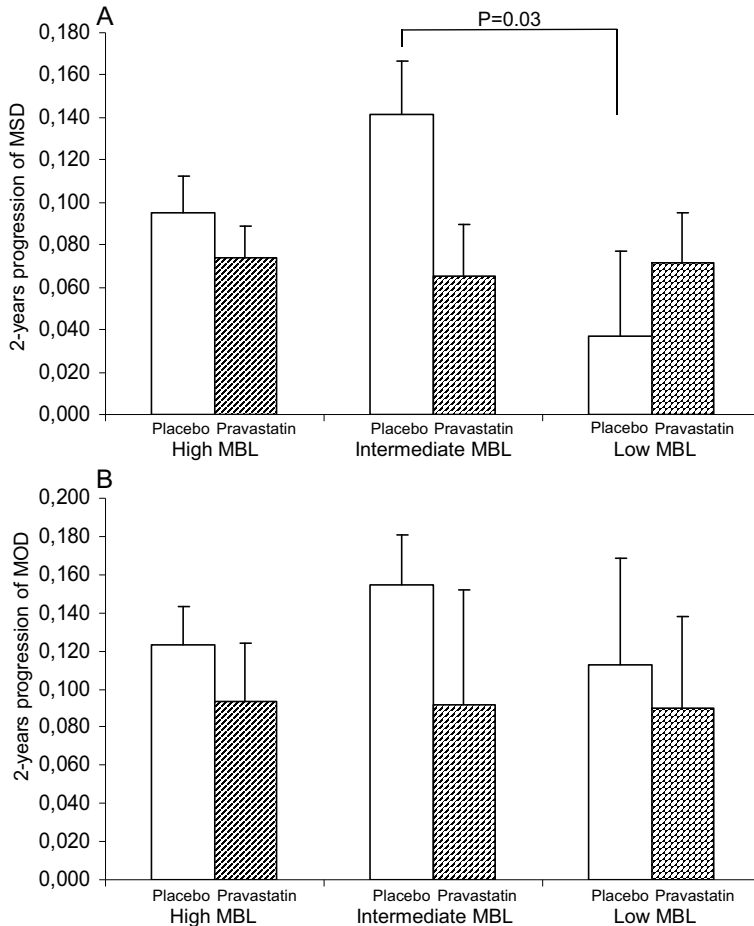


Figure 1. Mean \pm SEM progression of mean segment diameter (MSD) (Figure 1A) and minimal obstruction diameter (MOD) (Figure 1B) over 2 years by placebo and pravastatin in subjects with proven coronary artery disease divided in 3 groups of MBL2 secretor haplotypes (high, intermediate and low).

DISCUSSION

The present study suggests that there is no interaction between MBL2 haplotypes and pravastatin therapy in regard to the progression of CAD. In the past few years the relationship between MBL and atherosclerosis has not been settled in the literature. Both, low (3-9) and high (15-19) MBL2 secretor haplotypes, have been associated with the generation of atherosclerosis. This is the first study showing that the progression of coronary sclerosis does not depend on the MBL2 genotypes and that the treatment effect of pravastatin is not influenced by MBL2 haplotypes. If anything, the present data suggest that low MBL may protect from progression of coronary atherosclerosis compared to intermediate haplotypes. It is the intermediate group that seemed

to benefit most from intervention. Furthermore, we have shown that MBL2 haplotypes do not predict cardiovascular events in subjects with proven CAD in a 2 years follow up program.

The baseline MBL2 haplotype distribution was comparable with other studies conducted in Caucasians (12,13). This finding is interesting since all subjects in the REGRESS cohort had significant coronary atherosclerosis. If MBL2 secretor haplotypes were truly risk factors for atherogenesis, we should have found a higher frequency of one these secretor haplotypes in our group. Moreover, the fact that there were no differences in the history of myocardial infarction or for the family history for cardio-vascular disease strengthens the view that in the REGRESS cohort the MBL2 haplotypes do not represent a risk factor for atherosclerosis. However, due to the study design in which no subjects without coronary atherosclerosis were included, we were not able to compare baseline values of the REGRESS patients with non-atherosclerotic controls in a prospective study.

Activation of the complement pathways results in cleavage of complement component 3 (C3), which is central in these pathways (10). When MBL binds to its target (usually a microorganism), MBL-associated serine protease (MASP) functions as a convertase to clip C3 into C3a and C3b. Recent studies have described that elevated C3 is associated with CAD, insulin resistance, obesity, elevated fasting and postprandial triglycerides and disturbed postprandial free fatty acid handling (26-29). Furthermore, it has been shown that there is deposition of complement colocalized with CRP in atherosclerotic plaques (30,31) and that complement activation also plays a role in the induction of tissue damage after myocardial infarction (32). Therefore, the role of complement within atherosclerosis is well established. The role of MBL, however, is still subject of debate in the literature (3-9,15-19). Our results suggest that intermediate MBL2 secretor haplotypes are associated to increased progression and that these subjects might benefit the most from pravastatin treatment. However, the test for interaction between the MBL2 secretor haplotypes and therapy in both placebo and pravastatin groups showed that there was no relation for therapy and the change in progression of coronary atherosclerosis. One has to realize that the formation of activated C3 does not only depend on the MBL route, but can also be achieved by the alternative and classical pathways (10). All these data suggest that progression and generation of atherosclerosis may not be associated to MBL, but involves the complex system of complement with all its pathways and components. Future studies are needed to explore these mechanisms.

Our study has several limitations. Firstly, the REGRESS cohort only consisted of men. Keller et al found that increased serum MBL levels were a risk factor for future CAD in men but not in women (17). We did not measure MBL activity directly. However, it is known that the interplay between the promoter and structural gene variants controls the basal serum level and therefore the activity of MBL (12,13). An additional advantage was that genotyping also allowed us to study the mutations and their contribution to the progression of atherosclerosis. The second limitation in our study is the fact that rs7096206 and rs1800450 were not in Hardy-Weinberg equilibrium. This may be explained in two ways. Firstly, the locus being under selection,

embreeding or being analyzed in a population of multiracial origin. The latter is not the case since all the subjects were Caucasians. However, we can not rule out the possibility of the loci being under selection or embreeding. Secondly, there is a possibility of genotyping errors. Also this can not be completely ruled out, but the fact that the samples of known genotypes were included in every analysis as control and that the allele frequencies were fairly similar to the published data in all cases, is reassuring.

The final limitation of the study is the relative short length of follow-up of two years, which might be too short to detect subtle changes. Unfortunately, the original REGRESS study was designed as a two year trial. However, since the power of our study was above 80% for moderate effect-sizes of at least three of the MBL2 SNPs, we may conclude that there is no association between MBL2 SNPs and the MSD/MOD changes in 2 years of follow-up.

In conclusion, in men with proven coronary artery disease, the low, intermediate and high MBL2 secretor haplotypes do not evidently predict the progression of atherosclerotic burden and cardiovascular events in 2 years of follow up. Furthermore, there is no significant interaction between pravastatin therapy and MBL2 secretor haplotypes in regard to progression of coronary atherosclerosis.

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Disclosures

None declared.

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