Adding *MASP1* to the lectin pathway – leprosy association puzzle: hints from gene polymorphisms and protein levels.

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ABSTRACT

Background: Deposition of complement factors on *Mycobacterium leprae* may enhance phagocytosis. Such deposition may occur through the lectin pathway of complement. Three proteins of the lectin pathway are produced from the gene *MASP1*: Mannan-binding lectin-associated serine protease 1 (MASP-1) and MASP-3 and mannan-binding lectin-associated protein of 44 kDa (MAp44). Despite their obvious importance, the roles played by these proteins have never been investigated in leprosy disease. **Methodology:** We haplotyped five *MASP1* polymorphisms by multiplex sequence-specific PCR (intronic *rs7609662*G>A* and *rs13064994*C>T*, exon 12 3'-untranslated *rs72549262*C>G*, *rs1109452*C>T* and *rs850314*G>A*) and measured MASP-1, MASP-3 and MAp44 serum levels in 196 leprosy patients (60%, lepromatous) and 193 controls. **Principal findings:** Lower MASP-3 and MAp44 levels were observed in patients, compared with controls (P=0.0002 and P<0.0001, respectively) and in lepromatous, compared with non-lepromatous patients (P=0.008 and P=0.002, respectively). Higher MASP-3 levels occurred in controls carrying variants/haplotypes associated with leprosy resistance (*rs13064994*T, rs1109452_rs850314*CG* within *GT_CCG* and *rs850314*A*: OR=0.5-0.6, Pcorr=0.01-0.04). Controls with *rs1109452*T,* included in susceptibility haplotypes (*GT_GTG/GT_CTG*: OR=2.0, Pcorr=0.03), had higher MASP-1 and lower MASP-3 levels (P≤0.009). Those with *GC_CCG*, presented increasing susceptibility (OR=1.7, Pcorr=0.006) and had higher MAp44 levels (P=0.015). MASP-3 expression decreased in patients, compared with controls carrying *rs1109452_rs850314*CA* or *CG* (P≤0.02), which may rely on exon 12 CpG methylation and/or miR-2861/miR-3181 mRNA binding. **Conclusion:** Polymorphisms regulating MASP-3/MAp44 availability in serum modulate leprosy susceptibility, underlining the importance of lectin pathway regulation against pathogens that exploit phagocytosis to parasitize host macrophages.

Author summary

Since immemorial times, *Mycobacterium leprae* inflicts permanent injuries in human kind, within a wide symptomatic spectrum ranging from insensitive skin patches to disabling physical lesions. Innate resistance to this parasite is well recognized, but poorly understood. The complement system is one of the most important arms of the innate response, and several lines of evidence indicate that it may be usurped by the parasite to enhance its entrance into host cells. These include our recent work on genetic association of the disease with lectin pathway components and the complement receptor CR1, whose polymorphisms modulate susceptibility to infection and clinical presentation. Here, we add another pivotal piece in the leprosy parasite-host interaction puzzle: polymorphisms and serum levels of three different lectin pathway proteins, all encoded by the same gene, namely mannan-binding lectin-associated serine protease 1 (*MASP1*). We found lower levels of two of these proteins, MASP-3 and MAp44, in leprosy patients. Higher MASP-3/lower MASP-1 levels were associated with protective haplotypes, containing two side-by-side polymorphisms located in the exclusive untranslated region of MASP-3 exon 12, which may regulate exon splicing and/or translation efficiency. The associations revealed in this study reflect the pleiotropic nature of this gene. They further illustrate the complexity of the response mounted against the parasite, which places *MASP1* products in the regulatory crossroad between the innate and adaptive arms of the immunological system, modulating leprosy susceptibility.

INTRODUCTION

Leprosy is a chronic infectious disease caused by the obligate intracellular bacteria *Mycobacterium leprae*, irreversibly disabling about 8% of about 210,000 new cases every year, with Brazil ranking second in worldwide prevalence (1). To assist infection, *M. leprae* bacteria usurp complement activation to be opsonized and more readily phagocytosed into macrophages, one of their preferred host cells. Complement gene polymorphisms modulate

the abundance of the cascade components and susceptibility to leprosy. One way to mediate deposition of complement factors onto a surface is via the so-called lectin pathway of complement activation. One of the initiating proteins of this pathway is mannan-binding lectin, produced from the MBL2 gene. Since the first suggestion of a balancing selection operating on *MBL2* polymorphisms due to protection against mycobacterial diseases (2), much has been done investigating the possible roles played by genes of the lectin pathway of complement and their products on susceptibility to leprosy and tuberculosis (3) (4) (5) (6)(7) (8) (9) (10)(11). However, the exact role played by the lectin pathway components is still under investigation.

The very ancient origin of the complement system (12) (13), made it an ideal platform to coevolve with pathogens, like *M. leprae*, employing opsonins such as C3b to enter host phagocytic cells. In fact, the lectin pathway has long being recognized as favoring establishment of infection (2) (5) (4) (14) (6) (11). There is evidence that activation of complement modulate the course of the disease towards the Th1 or Th2 pole, from the paucibacillary tuberculoid, to the multibacillary lepromatous presentations of the disease, respectively (7) (3) (8) (15) (5). The lectin pathway of complement starts with the recognition of pathogen- or damaged/altered cell-associated patterns of carbohydrates or patterns of acetylated groups by pattern recognition molecules molecules (PRMs), i.e. collectins (Collectin-LK and mannose-binding lectin (MBL)) and the ficolins (FCNs), H-Ficolin (aka Ficolin-3), L-ficolin (Ficolin-2) and M-ficolin (Ficolin-1)) (16) (17) (18). These PRMs form circulating complexes with homodimers of MBL-associated serine proteases (MASPs) or MBL-associated proteins (MAps). Upon collectin/ficolin binding to a target, MASP-1 autoactivates and transactivates MASP-2, leading to cleavage of complement factors C2 and C4 in order to form the C3 convertase. This leads to enhanced deposition of the C3b molecule on the target, resulting in destruction by phagocytosis through complement receptors (CRs) or to the generation of membrane penetrating pores (membrane attack complexes) formed by C5b and the last complement components C6, C7, C8 and C9 (19) (20). Another result of complement activation is the generation of anaphylatoxins attracting cells to the site of activation. Another piece in the puzzle of the complement system is the alternative pathway, which is at least as old as the lectin

pathway. It becomes activated when the enzyme factor D is allowed to cleave other complement factors to allow for the generation of the alternative pathway C3 convertase, i.e. amplifying opsonization of microorganism. The enzyme Factor D becomes active when the enzyme MASP-3 cleaves pro-Factor D (21).

The *MASP1* gene (3q27.3) is highly pleiotropic as it by alternative pre-mRNA processing encodes the serine protease MASP-1 and MASP-3 and the protein MAp44 (aka MAP-1) (22) (23) (24) (25). These three proteins circulate in plasma in as homodimers in complexes with the PRMs mentioned above.

The substrate specificity of MASP-1 is described as being quite broad resembling some of the characteristics of thrombin and trypsin. Within the lectin pathway of complement activation MASP-1 can cleave MASP-2, and thus activating MASP-2 leading to C4 activation. But other enzymatic activities are also described. It has procoagulant activity, while it cleaves and activates Factor XIII and fibrinopeptide, generating fibrinopeptide B and attracting neutrophils to assist the coagulation cascade (26). It also activates carboxypeptidase B2, a molecule that prevents fibrinolysis and inactivates C3a and C5a anaphylatoxins (27). MASP-1 generates bradykinin from the cleavage of high-molecular-weight kininogen (28). It also cleaves PAR4 (protease-activated receptor 4) on endothelial cells and induces MAPKp38 (mitogen activated protein kinase protein 38) and NFkB (nuclear factor kappa-light-chain-enhancer of activated B cells) proinflammatory signaling (reviewed by (29) and (30)).

MASP-3 exclusively cleaves pro-factor D of the alternative pathway (31). This results in factor D cleavage of factor B complexed with C3b, creating the alternative pathway C3 convertase (reviewed by (32)). In the absence of MASP-3, only thrombin may possibly cleave some pro Factor D, but under circumstances of ongoing coagulation (33). As MASP-3 shares the same bindings sites on the PRMs with MASP-1 and MASP-2, it is also suggested to be able to compete on binding to the PRMs and thus to inhibit activation by the two other MASPs (18) (34). Some rare mutations in a highly conserved region of exon 12 of the *MASP1* gene, which is exclusive of MASP-3 and encodes the serine protease domain of this protein, cause the

3MC1 (Malpuech-Michels-Mingarelli-Carnevale) syndrome, pointing to an important role in ectodermal development (35).

MAp44 may also compete with MASPs for binding sites on the PRMs and in such manner regulate MASP mediated complement activation, i.e. it does have the ability of displacing MASP-1 and MASP-2 from within the collagenous stalks of the PRMs (24) (36). It is highly expressed in the heart, which suggest that it may reduce the damage which may occur with uncontrolled activation of the lectin pathway, after ischemia-reperfusion injury (25).

The role of MASPs in the establishment of infections and in leprosy progression is still poorly understood. Low MASP-2 levels, as well as *MASP2* polymorphisms associated with low MASP-2 production, were associated with increased susceptibility to leprosy (6). Low MBL levels and corresponding *MBL2* polymorphisms, in contrast, were associated with increased resistance (7) (3), and higher FCN-3 levels were more frequent in leprosy patients than in controls (9). It has also been suggested that complement receptor CR1 and CD91/calreticulin bind the collagenous chains of collectins and ficolins deposited on pathogens or altered cells, leading to their internalization, but that MASPs and MAps compete with this binding site, preventing this recognition (37) (38). CR1 binds opsonized *M. leprae* to enter the cell (39), and may use C3b and collectins/ficolins. Interestingly, we recently found polymorphisms of the *CR1* gene associated with leprosy, as well as a negative correlation between the anti-inflammatory soluble CR1 and pro-inflammatory MBL levels, probably preventing inflammation (10).

Given this context, we investigated whether *MASP1* gene variants and products are associated with susceptibility to leprosy and to the different clinical forms of the disease. We aim at providing a better understanding of the immunological clinical spectrum of leprosy and of the role played by the lectin pathway in mycobacterial infections.

MATERIAL AND METHODS

Subjects and Samples

We included leprosy patients comprising of a total of 196 individuals with 138 being consecutive outpatients from the Clinical Hospital of the Federal University of Paraná (HC-UFPR) and 58 inpatients from the Sanitary and Dermatologic Hospital of Paraná both in Curitiba, Brazil. This study was conducted according to the Declaration of Helsinki. The local medical ethics committee of the HC-UFPR approved the study (protocol 497.079/2002–06, 218.104 and 279.970) and all subjects signed a written informed consent. Patients were diagnosed based on clinical and histopathological features and classified according to Ridley and Jopling criteria (40). The control group comprised of 214 blood donors from the Hemepar and HC-UFPR blood banks and were from the same socioeconomic, ethnic and geographic background. Patients and controls were defined as Euro-, Afro-Brazilians or Amerindians, based on physical characteristics and ancestry information. This means 9% and 5% average sub-Saharan African and Amerindian ancestry respectively, for the former, and at least 40% of African and 6% of Amerindian ancestry for the latter, based on HLA genotyping for South Brazilian populations classified in the same way (41) (42) (Table 1). Blood was collected with, or without for serum collection, anticoagulant ethylenediaminetetraacetic acid (EDTA) and DNA was extracted from peripheral blood mononuclear cells through commercial kits (Qiagen GmbH, Hilden, Germany and GFX™ Genomic Blood DNA Purification Kit, GE Healthcare, São Paulo, Brazil).

Table 1. Clinical and demographic description of controls and leprosy patients.

Parameters	Controls	Patients	Exact P value
N	214	196	-
Age average [Min-Max]	38.17 [18-61]	51.31 [18-94]	<0.0001
Male (%)	116 (54.2)	119 (60.7)	0.195

Ethnical background (%)*			0.70
Euro-Brazilian	176 (82.2)	158 (80.6)	-
Afro-descendant	34 (17.3)	36 (18.6)	-
Amerindian	4 (2.1)	2 (1.1)	-
Clinical Form (%)			
Lepromatous	n.a.	118 (60.2)	n.a.
Borderline	n.a.	27 (13.7)	n.a.
Tuberculoid	n.a.	18 (9.2)	n.a.
Indeterminate	n.a.	10 (5.1)	n.a.
Non-specified	n.a.	23 (11.7)	n.a.

Table 1. Clinical and demographic description of controls and leprosy patients.

n: number of individuals; na.: not applicable

*: Ethnic background based on physical characteristics and ancestral information, corroborated by HLA genotyping of South-Brazilians classified in the same way (Probst et al. 2000, Braun-Prado et al. 2000).

MASP1 genotyping

A sequence-specific multiplex amplification method (multiplex PCR-SSP) was optimized in order to haplotype five single nucleotide polymorphisms (SNPs): *rs7609662*G>A* and *rs13064994*C>T* in intron 1 and *rs72549262*G>C*, *rs1109452*C>T* and *rs850314*G>A* in exon 12 within the 3' untranslated (UTR) region (reference sequence: ENST00000337774.9). We amplified a 730 bp fragment specific for rs7609662 and rs13064994 in intron 1 and co-amplified a 365 bp fragment specific for rs72549262 and rs1109452+rs850314 (both are adjacent SNPs) in exon 12, all in a batch of four low-cost reactions, as previously described for *MASP2* (6). As a control for the amplification quality, we co-amplified a 500 bp fragment in every single reaction, corresponding to exon 8 of the Ficolin 2 gene (*FCN2*) by adding two

generic primers (**Table 2**). The protocol starts with a denaturation step of 3 min at 96C, followed by 35 cycles of 20 sec at 94C for denaturation, 30 sec for primer annealing at variable temperatures (see below), and 30 sec DNA extension at 72C, concluding with 1 min and 30 sec at 72C or extension. We used three different annealing temperatures according to previously published "touch-down" protocol: the 10 first cycles at 61C, followed by 10 cycles at 59C and 15 cycles at 57C. The haplotypes defined by these five SNPs, were identified by the presence or absence of specific bands in agarose gel, after electrophoresis.

Table 2. *MASP1* sequence-specific primers and fragment size.

Forward Primers		Reverse Primers	
Intron 01			
MASP1 rs7609662_Af	5' ATATTTGTTTCATATGTTTGAAACC**A** 3'	*MASP1* rs13064994_Cr	5' TTCTTAAACCAATCTGT
MASP1 rs7609662_Gf	5' ATATTTGTTTCATATGTTTGAAACC**G** 3'	*MASP1* rs13064994_Tr	5' TTCTTAAACCAATCTGT
Exon 12			
MASP1 rs72549262_Cf	5' CCCTCTCTCTTAGTGTGAT**C** 3'	*MASP1* rs1109452_Tr	5' CGACTAAGTCCCCATAT
MASP1 rs72549262_Gf	5' CCCTCTCTCTTAGTGTGAT**G** 3'	*MASP1* rs1109452_Cr	5' CGACTAAGTCCCCATAT
		MASP1 rs850314_Ar	5' CGACTAAGTCCCCATAT

Each primer is named after the SNP it amplifies, f: forward; r: reverse. In bold: variant nucleotides; bp: base pairs.

MASP-3 and MAp44 levels assays

Serum concentrations of MASP-3 and MAp44 were determined by time-resolved immunofluorimetric assays (TRIFMA) for 142 and 145 patients, respectively, and 116 controls, as previously described (24). Briefly, samples were diluted in binding buffer, 40-fold for MAp44 detection and 100-fold for MASP-3, and incubated in microtiter wells coated with a monoclonal antibody. The bound protein is detected by a specific biotin-labeled monoclonal antibody, which is then subsequently detected by europium-labeled streptavidin. The provided signal is measured by time-resolved fluorometry. Four internal controls were added to each assay plate in both assays.

MASP-1 levels assay

The time-resolved immunofluorimetric assay for MASP-1 is an inhibition assay, where circulating MASP-1 in the sample inhibits the binding of an anti-MASP-1 antibody to a surface coated with a fragment of MASP-1, as previously described (36). Briefly, diluted serum samples of 141 patients and 116 controls, 60-fold in binding buffer, were incubated with an equal volume of diluted rat anti-MASP-1 antibody for approximately an hour and then added to the coated microtiter wells. Bound rat anti-MASP-1 were detected with biotinylated rabbit anti-rat-Ig followed by europium-labeled streptavidin, where bound europium is measured by time-resolved fluorometry. Four internal controls were also added to each plate for this assay.

Statistics

Genotype, allele and haplotype frequencies were obtained by direct counting. The expectation maximization (EM) algorithm was used to calculate maximum likelihood estimates of intron 1 – exon 12 haplotype frequencies, while taking into account phase ambiguity. The hypothesis of Hardy–Weinberg equilibrium and of homogeneity between allelic distributions (exact test of population differentiation of Raymond and Rousset) was also evaluated with the ARLEQUIN software package version 3.1 (http://anthro.unige.ch/arlequin/). Protein levels were compared between the groups using nonparametric Mann-Whitney/Kruskal–Wallis tests (since their distribution did not pass Shapiro-Wilk normality test), using Graphpad Prism 5.01 (GraphPad Software, La Jolla, CA). The reduced model of multivariate logistic regression was used to adjust results for demographic factors; age, sex (factors that might influence protein levels (43)) and ethnic group, as well as for previously published MASP-2 levels, *MBL2, MASP2, FCN1*, *FCN2* and *FCN3* genotyping results (44) (3) (9) (45) using STATA v.9.2 (Statacorp, TX, USA). The P values obtained with multiple comparisons in the association studies were corrected with the Benjamini-Hochberg method.

RESULTS

Protein serum levels in the Southern-Brazilian patients and controls were within the range reported for a Danish population (34) (24). We found strong evidence for an association between MASP-3 and MAp44 serum levels and leprosy. We also identified a genetic association between MASP-1 and MASP-3 serum levels and *MASP1* polymorphisms, composing haplotypes associated with increased resistance and susceptibility to leprosy. The results are described in detail below.

MASP-3 and MAp44 levels are associated with leprosy per se and lepromatous leprosy

Leprosy patients presented lower MASP-3 levels (median 4,488 [1,722-14,634] ng/mL), than controls (median 5,575 [2,149-12,579] ng/mL) (Mann-Whitney P<0.001). In fact, the frequency of individuals with more than 5,500 ng/mL circulating MASP-3 in serum was higher among controls: 51.7% or 60/116, compared with 31.7% or 45/142 in patients, independently of age and sex distribution (logistic regression OR=0.51 [95%CI=0.28-0.92] P=0.026) (S1 Fig). MASP-3 levels were even lower in lepromatous patients, who present numerous severe lesions with multiple bacilli and an exacerbated Th2 immune response. In these severely affected, often disabled patients, the median of MASP-3 levels was 4,209 [1,722-11,244] ng/mL, compared with 5,334 [2,021-14,634] ng/mL in patients with the other clinical forms (Mann-Whitney P=0.0083). Individuals with MASP-3 levels higher than 5,500 ng/mL were also much more frequent among non-lepromatous (50% or 18/36), compared with lepromatous patients (26.8% or 26/97), independently of age and sex distribution (logistic regression OR=0.38 [95%CI=0.16-0.90], P=0.028).

MAp44 levels followed a similar trend, but with a more conspicuous difference. Leprosy patients also presented lower MAp44 levels (median 1,715 [719-4,843] ng/mL in patients vs. median 2,330 [1,140-4,927] ng/mL in controls; Mann-Whitney P<0.0001) (Fig 1). As in the case of MASP-3, individuals with MAp44 levels higher than 2,300 ng/mL were much more frequent among controls: 50.9% or 59/116, compared with 22.1% or 32/145 in patients, independently of age and sex distribution (logistic regression OR=0.26 [95%CI=0.14-0.49] P<0.0001) (S1 Fig). This pattern was also followed by lepromatous, compared with non-lepromatous patients: MAp44 median 1,646 [719-4,843] ng/mL vs. median 1,995 [985-4,359] ng/mL, respectively (Mann-Whitney P=0.0021) (Fig 2). Individuals with MAp44 levels higher than 2,300 ng/mL were also much more frequent among non lepromatous (36.1% or 13/36), compared with lepromatous patients (15.5% or 15/97), again independent of age and sex distribution (logistic regression OR=0.34 [95%CI=0.13-0.89], P=0.023).

Figure 1: MASP-1 (A), MASP-3 (B) and MAp44 (C) serum levels in controls and leprosy patients. Data shown with medians and interquartile ranges and Mann-Whitney P values. Open and closed symbols represent controls and patients, respectively.

Figure 2: MASP-1 (A), MASP-3 (B) and MAp44 (C) serum levels in non-lepromatous and lepromatous patients. Data shown with medians and interquartile ranges and Mann-Whitney P values. Open and closed symbols represent controls and patients, respectively.

In contrast, MASP-1 levels did not differ between patients and controls (median 7,036 [2,350-14,109] ng/mL vs. 6,207 [2,521-16,624] ng/mL, respectively; Mann-Whitney P=0.173) or among the lepromatous patients and those with the other clinical forms (Mann-Whitney P=0.603) (Figs 1 and 2).

MAp44 levels correlated significantly, but weakly with the other two serine proteases (MASP-1: R=0.21 in patients, Spearman P<0.05; MASP-3: R=0.05 in patients, R=0.36 in controls, both with Spearman P<0.0001). There was no correlation between MASP-1 and MASP-3 levels or MAp44 and MASP-1 levels in controls (S1 Fig). These results were expected, according to former reports (34) (36).

MASP1 polymorphisms and haplotypes associated with leprosy

The allele frequencies for the investigated *MASP1* SNPs did not differ from Iberians (who contributed most to the Southern-Brazilian population), as well as from other Europeans, according to the 1000 Genomes project (exact test of population differentiation) (2014). (Table 3). We identified three intron 1 haplotypes: *AC*, *GC* and *GT*. The *GC* combination accounted for more than half of all intron 1 haplotypes in the investigated groups. There were also four exon 12 haplotypes: *CCA*, *CCG*, *CTG* and *GTG*. Of these, *CCG* was the most common, but none of the others presented less than 5% frequency. The genotypic distributions of these haplotype combinations were in Hardy and Weinberg equilibrium, excepting the distribution of exon 12 haplotypes in patients (P=0.01). Haplotype distribution further differed between leprosy patients and controls (exact test P=0.016), as well as between lepromatous patients and controls (exact test P=0.023), but not between lepromatous and non-lepromatous patients. In accordance, there was no association of *MASP1* alleles/haplotypes/genotypes with the lepromatous clinical form of the disease. Furthermore, no associations with the disease occurred with the two variants located in intron 1. All other associations were still significant after correction for multiple comparisons (Pq value) and for age (the only demographic factor that remained associated with the disease in the reduced logistic regression model).

Linkage disequilibrium between the intron 1 and exon 12 alleles resulted in a total of twelve different *MASP1* haplotypes in leprosy patients and thirteen in controls, among which those with frequencies higher than 10% were *GC_CCG,* followed by *GT_CCG, GC_CTG, GC_CCA* and *AC_CCG.* Three of them were associated with leprosy, independently of any other demographic factor (**Table 3**).

Variants	Iberian	Controls	Patients	Lepromatous	Others	Model	Patie Cont
	% (n)	% (n)	% (n)	% (n)	% (n)		
Total genotypes	100 (107)	100 (214)	100 (196)	100 (118)	100 (55)		
rs7609662 (*c.5+2718G>A*)							OR [
A	13.6 (29)	14.1 (60)	14.6 (58)	13.5 (32)	13.6 (15)		ns
G/G	74.8 (80)	74.3 (159)	72 (141)	72.8 (86)	74.5 (41)		ns
G/A	23.4 (25)	23.3 (50)	27 (53)	27.2 (32)	23.6 (13)		ns
A/A	1.9 (2)	2.3 (5)	1 (2)	0 (0)	1.8 (1)		ns
rs13064994 (*c.6-2172C>T*)							
T	26.9 (49)	28.5 (123)	27.8 (109)	30.1 (71)	27.3 (30)		ns
C/C	54.2 (58)	50 (107)	50.5 (99)	45.7 (54)	54.5 (30)		ns
C/T	33.6 (36)	43 (92)	43.3 (85)	48.3 (57)	36.3 (20)		ns
T/T	12.1 (13)	7 (15)	16.1 (12)	3 (7)	9.1 (5)		ns
rs72549262 (*c.1304-5229C>G*)							
G	8.9 (19)	11.3 (48)	7.7 (30)	6.8 (16)	5.4 (6)		ns
C/C	82.2 (88)	80.8 (173)	87.2 (171)	84.7 (100)	91 (50)		ns
C/G	17.8 (19)	15.8 (34)	10.2 (20)	10.2 (12)	7.3 (4)		ns
G/G	0(0)	3.2 (7)	2.5 (5)	1.7 (2)	1.8 (1)		ns
rs1109452 (*c.1304-4903C>T)*							
T	25.2 (54)	33.5 (143)	33.7 (132)	35.6 (84)	30 (33)		ns
C/C	57.9 (62)	46.3 (99)	44.9 (88)	41.5 (49)	51 (28)		ns
C/T	33.6 (36)	40.6 (87)	42.8 (84)	45.7 (54)	38.2 (21)		ns
T/T	8.4 (9)	13.1 (28	12.2 (24)	12.7 (15)	10.9 (6)		ns
rs850314 (*c.1304-4902G>A*)							
A	32.7 (70)	19.9 (86)	15.3 (60)	13.1 (31)	18.1 (20)		ns
G/G	47.7 (51)	64 (137)	73 (143)	75.4 (89)	71 (39)		ns
G/A	39.3 (42)	32.2 (69)	23.4 (46)	22.9 (27)	21.8 (12)	Dom	0.60 [
A/A	13.1 (14)	3.7 (8)	3.5 (7)	1.7 (2)	7.3 (4)		ns
Intron 1_Exon 12 Haplotypes							
GT_GTG *		0.7 (3)	1.8 (7)	1.7 (4)	0.9 (1)	Addit	2.19 [
GT_CTG		6.1(26)	9.2 (36)	10.6 (25)	9.1 (10)	Dom	2.01 [
GT_CCG		16.6 (71)	12.2 (48)	12.7 (30)	11.8 (13)	Dom	0.52 [
GT_CCA		5.1 (22)	4.6 (18)	5.1 (12)	5.4 (6)		ns
GC_GTG		49.3 (40)	5.6 (22)	7.2 (17)	4.5 (5)		ns
GC_CTG		13.7 (59)	14 (55)	13.6 (32)	12.7 (14)		ns
GC_CCA		14.0 (60)	9.4 (37)	6.7 (16)	11.8 (13)	Dom	0.48 [
GC_CCG		20.3 (87)	28.6 (112)	28.8 (68)	30 (33)	Addit	1.70 [
AC_CCA		2.3 (10)	1.2 (5)	1.2 (3)	0.9 (1)		ns
AC_CTG		0.7 (3)	2.8 (11)	2.5 (6)	1.8 (2)		ns
AC_GTG		1.1 (5)	0.2 (1)	0 (0)	0.9 (1)		ns
AC_CCG		9.8 (42)	10.2 (40)	9.7 (23)	10 (11)		ns

Table 3. Association of *MASP1* variants and haplotypes with leprosy. The intron 1 and exon 12 haplotypes were unambiguously build with sequence-specific amplification. The phase between them (symbolized by "_") was inferred using the expectation maximization algorithm. Official SNP nomenclature is given within parenthesis for the longest cDNA, corresponding to the mRNA transcript encoding MASP-1: ENST00000337774.9. Addit: Additive association model, which tests the hypothesis that homozygosity and heterozygosity for the minor allele are associated with leprosy (either with protection or with susceptibility), but homozygosity is stronger associated, than heterozygosity. Dom: Dominant association model, which tests the hypothesis that the carrier status of the minor allele (regardless if homozygous or heterozygous) is associated with leprosy (either with protection or with

susceptibility) All associations were corrected for age, which was the only demographic factor that remained associated in the reduced model of logistic regression. *: *GT_GTG* + *GT_CTG* association. q*: Benjamini-Hochberg corrected p values; ns: not significant; OR: odds ratio; CI: confidence interval.

The strongest association was found with the most frequent *GC_CCG* haplotype, which was associated with an additive (allele-dosage) susceptibility effect (OR=1.70 [95%CI=1.21–2.40], P<0.005). This is explained by a higher frequency of *GC_CCG* homozygotes and of *GC_CCG* heterozygotes among leprosy patients (21/196 or 10.71% and 70/196 or 35.71%), than among controls (16/214 or 7.48% and 55/214 or 25.7%), respectively. A dominant, age-dependent effect towards leprosy susceptibility was associated with carrying the less frequent *GT_CTG* haplotype (OR=2.01 [95%CI=1.06–3.83], P=0.033). In other words, older individuals with this haplotype seem more prone to develop leprosy, if infected: there was 35/196 or 17.9% leprosy patients with *GT_CTG*, of which 26/35 or 74.3% with at least 40 years of age. In comparison, only 24/214 or 11.21% controls carried this haplotype, of whom only a third (8/24 or 33.3%) were at their forties or older. Notwithstanding, the same analysis with either *GT_CTG* and/or another uncommon haplotype with *GT* in intron 1, namely *GT_GTG,* turned the association age-independent (OR=2.19 [95%CI=1.18-4.03], P=0.012). Thus, age-dependency has a rather weak effect or may simply result from sampling bias.

In contrast, two haplotypes were associated with protection against leprosy. Among them, *GC_CCA* was associated with a dominant protective effect (OR=0.48 [95%CI=0.29–0.82], P=0.008). This means that carriers of this haplotype were much more frequent among controls (59/214 or 27.6%), than among leprosy patients (36/196 or 18.4%). Similarly, controls presented a higher frequency of *GT_CCG* carriers (71/214 or 33.2%), compared with leprosy patients (43/196 or 21.9%). This haplotype was also associated with a dominant resistance effect against the disease (OR=0.53 [95%CI=0.32–0.86], P=0.011) (**Table 3**).

MASP1 polymorphisms associated with protein serum levels

Although there was no association between the intron 1 *rs7609662*G>A* variant and *MASP1* protein products, the neighboring *rs13064994*C>T* polymorphism was associated with MASP-3 serum concentrations. Healthy carriers with the *rs13064994*T* variant presented higher MASP-3 levels, than *C/C* homozygotes (medians 6,022 [2,286-11,820] ng/mL vs. 5,086 [2,149-12,580] ng/mL, respectively, P=0.0103). This difference disappeared among leprosy patients, whose MASP-3 concentrations reached lower levels, independent of the genotype (medians 4,557 and 4,228 ng/mL, respectively) (Fig 3A).

Figure 3: Association between variant alleles and MASP levels. (A) rs13064994 in intron 1 and MASP-3; **(B)** rs850314 in exon 12 and MASP-3; **(C)** rs1109452 in exon 12 and MASP-3; **(D)** rs1109452 in exon 12 and MASP-1.
Data shown with medians and interquartile ranges and Mann-Whitney P values. Open and closed symbols represent controls and patients, respectively.

Regarding the exon 12 variants, there was no association with the rs72549262 variant. However, in accordance with the associated effect of the intron 1 rs13064994 polymorphism, controls with the minor *rs850314*A* allele of exon 12 presented higher MASP-3 levels, than *G/G* homozygotes (6,373 [2,286-11,820] ng/mL vs. 5,450 [2,149-11,480] ng/mL, P=0.0342). This difference was no longer noticeable among patients, whose MASP-3 levels were generally lower (medians 4,500-4,554 ng/mL) and seemed no longer to be under the same genetic control (Fig 3B). In contrast, carriers of the minor *rs1109452*T* allele presented lower MASP-3 levels in controls, although they did not differ between healthy and diseased carriers (Fig 3C). Contrary to MASP-3 levels, MASP-1 serum concentration of *rs1109452*T* carriers were higher than in *C/C* homozygotes, independent of the disease (Fig 3D).

The adjacent exon 12 *rs1109452*C* and *rs850314*A*, as well as *rs1109452*T* and *rs850314*G* variants, occur in absolute linkage disequilibrium. The *CA, CG* and *TG* haplotype combinations did not present any association with MASP-1 and MAp44 levels (Figs 4A and 4C), although leprosy patients presented consistently lower MAp44 levels, regardless of the

exon 12 genotype (Fig 4C). Healthy individuals with the *CA*, as well as with the *CG* haplotype, presented higher MASP-3 concentrations than those with the *TG* haplotype (*CA* median 6,521 [2,286-11,820] ng/mL and *CG* median 5,858 [2,286-11,820] ng/mL vs *TG* median 5,071 [2,149-8,941] ng/mL). In contrast to individuals with the *CA* and *CG* haplotypes, baseline levels of healthy individuals carrying *TG* do not differ from those with leprosy (Fig 4B).

Figure 4: Association between haplotypes with the rs1109452 and rs850314 adjacent exon 12 variants and levels of *MASP1* products. Data shown with medians and interquartile ranges and Mann-Whitney P values. Open and closed symbols represent controls and patients, respectively. *CA*+: carriers of the *rs1109452*C* and *rs850314*A* variants. *CG*+*:* carriers of the *rs1109452*C* and *rs850314*G* variants. *TG*+: carriers of the *rs1109452*T* and *rs850314*G* variants. Unless if otherwise stated, comparisons were made with Mann-Whitney test.

Healthy individuals carrying the *GT_CCG* haplotype presented higher MASP-3 levels than those without it (median: 6,131 [2,286-11,820] ng/mL vs. 5,148 [2,149-12,580] ng/mL), a difference no longer noticed among leprosy patients (Fig 5A). Similarly, controls with the *GC_CCG* haplotype, but not patients, presented higher MAp44 levels (median 2,581 [1,355-4,927] ng/mL vs. 2,272 [1,140-4,068] ng/mL) (Fig 5B).

Figure 5: *MASP1* haplotypes associated with (A) MASP-3 and (B) MAp44 levels. Data shown with medians and interquartile ranges and Mann-Whitney P values. Open and closed symbols represent controls and patients, respectively. + with the haplotype, - without the haplotype.

Discussion

Parasitic *Mycobacteria* species are known to usurp and efficiently evade the host defense response (reviewed by (46) & (47)). However, investigating the immune response elicited by *M. leprae* remains a particular

challenge, due to its extreme dependence on the human host. Genetic disease association studies shed light on a wide range of aspects from the onset of infection to disease cornification, by uncovering genes whose protein products may play pivotal roles in this pathology (48). This has been the case for several genes of the lectin pathway of complement; those encoding PRMs, *MBL2* (3) (4) (14), *FCN1* (5), *FCN2* (4) and *FCN3* (9) and the serine protease *MASP2* (6) and the possible receptor for MBL encoded by *CR1* (10). The evaluation of complement protein levels adds highly relevant information to this picture, as an indirect measure of gene expression, complement activation and consumption. Since the seventies, these measurements have been done for leprosy disease (49) (8) (7), with results currently supported by transcriptome studies (50). In the present investigation, we finally added *MASP1* polymorphisms and protein products, as one important piece of the initiation complexes of the lectin pathway to the association of complement with leprosy disease.

To understand the possible roles of *MASP1* products in the disease, it is important to keep in mind two prevailing hypotheses that may explain the role of complement proteins in leprosy disease. First, they increase infection success by improving opsonization and phagocytosis of *M. leprae* by the host macrophage cells. Second, they increase inflammation after the disease is established, leading to more severe tissue damage.

Regarding the first hypothesis, it may be argued that any variant that reduces the rate of opsonin deposition would be protective, whereas any variant that increases opsonization would enhance susceptibility. According to this, one would expect that high MASP-1 levels would aid *M. leprae*'s entrance into host cells, whereas high MASP-3/MAp44 levels would block activation of the lectin pathway and reduce phagocytosis of the bacteria (although MASP-3 may also activate the alternative pathway). In fact, higher MASP-3 and MAp44 levels were characteristic for healthy individuals, although the expected effect was not seen for MASP-1 (Fig 6). With respect to the second hypothesis, it is expected that variants that reduce complement activation would (again) play a protective role. Indeed, we found a clear-cut difference between patients, with higher MASP-3/MAp44 levels more prevalent among those, less severely affected. Since it is known from former

studies that Dapsone and Clofazimine treatment (used by the patients in this study) does not interfere with complement availability and function (51) (52), it may be assumed that lower MASP-3 and MAp44 levels among patients, especially among those with the most severe lepromatous condition, are genetically determined (Fig 6).

Fig 6: Proposed roles for *MASP1* products and polymorphisms in susceptibility to *M. leprae* infection. (A) Collectins (e.g. MBL) or ficolins (e.g. FCN-3) recognize pathogen-associated molecular patterns (PAMPs), composed of sugar/acetylated groups on *M. leprae.* MASP-2 (not depicted in this image) and MASP-1 homodimers complexed with them activate the lectin pathway of complement, whereas MASP-3 may activate the alternative pathway. Both pathways lead to C3b-opsonization and CR1-mediated internalization of the pathogen. **(B)** Healthy individuals with *rs1109452* and *rs850314* CA or CG haplotypes express higher MASP-3 levels. Higher MASP-3 and MAp44 levels were also associated with resistance against the disease. **(C)** CpG methylation at the *CG* haplotype in exon 12 may impair mRNA transcription, spliceosome assembly and mRNA processing. Reduced MASP-3 levels may also result from the differential recognition of *CA* and *CG* haplotypes by miRNAs (miR-2861 and miR-3181, respectively). **(D)** Individuals with TG haplotypes present lower baseline MASP-3 levels. Lower MASP-3 and MAp44 levels seem to predispose to the infection, possibly by optimizing opsonin coverage of the parasite.

There are numerous polymorphisms in the *MASP1* gene that may interfere with gene expression, some of which had been formerly investigated by others (53) (17). We chose to investigate two SNPs located in a regulatory region of intron 1, which may interfere with the production of all three *MASP1* proteins, and three in exon 12, which is exclusive of MASP-3 and may uniquely affect the expression level of this protein. None of them had been previously investigated.

*rs7609662*A* in intron 1 is associated with higher *MASP1* mRNA levels in several tissues (https://gtexportal.org/home/snp/rs7609662), but we did not identify this effect at the protein level. The *rs13064994*T* had the opposite effect (https://gtexportal.org/home/snp/rs13064994) on *MASP1* mRNA expression. We found an association of this allele with higher MASP-3 protein levels, but only in healthy individuals. The absence of a clear correlation between mRNA levels and protein concentration in serum is not unexpected,

since former analyses did not consider different *MASP1* transcripts, and stability of mRNA in cytoplasm may be greatly affected by regulatory mechanisms that were not accounted for in previous transcriptomic analyses.

All exon 12 variants (*rs72549262*G>C*, *rs1109452*C>T* and *rs850314*G>A*) are located within the 3' untranslated region. Those two most downstream (rs1109452 and rs850314) are adjacent to each other, and *CG* represents the most ancestral combination. Thus, the minor alleles *rs1109452*T* and *rs850314*A* disrupt a 5'CpG3' site (where "p" means the phosphodiester bond between *rs1109452*C* and *rs850314*G*). The cytosine of this CpG site was found methylated in the brain (54), but not in cell lines from liver and female reproductive tissue, where MASP-3 mRNA production is highest (https://gtexportal.org/home/gene/MASP1). DNA methylation in alternatively spliced exons may modulate exon inclusion (55).

Furthermore, the *CA* and *CG* combinations are miRNA targets, as predicted *in silico* using targetScan7.1 (REF), and may reduce MASP-3 translation. Thus, one would expect that any nucleotide substitution at these loci would modify gene expression, depending on specific regulatory requirements of the cell type, developmental stage, physiological and immunological responses. In fact, both adjacent polymorphisms were associated with MASP-3 (in the case of rs1109452, even MASP-1) levels. However, the predicted down-regulating effects either of CpG methylation and/or *CA/CG* miRNA binding on MASP-3 levels, were restricted to leprosy patients. In the disease, MASP-3 levels of *CA* or *CG* carriers dropped to the same concentration found in *TG* carriers, who presented the lowest MASP-3 levels, independent of the disease. Interestingly, among the miRNAs predicted to recognize these polymorphic sites, none bind *TG,* but miR-3181 preferentially recognizes *CG* and miR-2861, *CA.* Both are expressed in the liver (56), with miR-2861 being up-regulated by interleukin 6 (57), a proinflammatory cytokine with a pivotal role in leprosy disease (58). It is thus conceivable that these regulatory mechanisms operate after disease establishment and activation of the acute phase response (Fig. 6).

Refining the association analysis to the haplotype level, allowed us to identify the *GT_CCG* and *GC_CCA* haplotypes (containing the previously

mentioned *rs850314*A* variant) associated not only with higher MASP-3 levels, but also with higher protection against the disease. Higher MASP-3 levels may avoid initiation of bacterial colonization due to competition with MASP-1 and MASP-2 for binding sites of recognition molecules - blocking the lectin pathway, and/or by competition with binding sites on complement receptors, blocking phagocytosis.

Yet the *GC_CCG* haplotype, associated with leprosy susceptibility, was associated with higher MAp44 serum concentrations. In contrast with MASP-3, however, MAp44 serum levels did not associate with the investigated SNPs, which may suggest other causal variants in linkage disequilibrium with *GC_CCG*, not investigated in this study. In fact, Ammitzboll et al. (2013) list several variants that may modulate MAp44 levels. Furthermore, other factors than those regulating MASP-3 may fit in the present scenario, where MAp44 levels are higher in controls, compared to patients, and in non-lepromatous patients, compared to the more severely affected lepromatous patients.

Beside *GC_CCG,* the haplotypes *GT_CTG* and *GT_GTG* also present at least an additive effect increasing almost twice susceptibility to the disease. They were not associated with protein levels, although harboring the *rs1109452*T* polymorphism, found associated with higher MASP-1 and lower MAp44 levels. Thus, protein levels shall not be held solely responsible for the association of *MASP1* products with the disease. Beside the pleiotropic nature of the *MASP1* gene itself, the investigated polymorphisms may have effects far beyond those affecting *MASP1*, and other variants linked with those that compose the associated haplotypes, may present epistatic and/or unsuspected pleiotropic effects that affect susceptibility to the disease. In fact, the variants investigated in this study have been recently associated with expression levels of neighboring genes as the ribosomal protein-encoding gene *RPL39L* and the odorant receptor transporters *RTP1, RTP3* and *RTP4* (https://gtexportal.org/home/gene/MASP1 and Immunpop browser). Among them, RTP4 is strongly up-regulated by interferon I, a cytokine known to suppress an adequate cellular response driven by interferon type II against *M. leprae* (59).

Thus, MASP-3/MAp44 blockage of the lectin pathway may not be the only explanation for resistance, since expression levels of neighboring genes

664 may be regulated by noncoding polymorphisms investigated in this study.
665 Although interpreting the evidence is not straghtforward, it certainly fosters
666 more investigations on the role played by *MASP1* products in the resitance
667 against mycobacterial infections and its more severe forms. In particular,
668 MASP-3 and MAp44 may be evaluated as new therapeutic agents against
669 leprosy infection and against polarization to lepromatous disease.
670
671

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677

678

679 Supplementary data

680

681 **S1 Fig: Correlations between MASP-1, MASP-3 and MAp44 serum levels in leprosy**
682 **patients (A-B) and healthy controls (C-D)**
683 Linear regression fit, P and R values are shown.
684
685 **S1 Table. Masp-1, Masp-3 and MAp44 levels in patients an controls.**
686 n: number of individuals; *: mean protein levels in ug/mL showing: median[IQR] **Levels
687 confering protection against Leprosy infection. Within brackets: minimum and maximal
688 values. [a]: Patients presenting all other forms except Lepromatous and Non-specified
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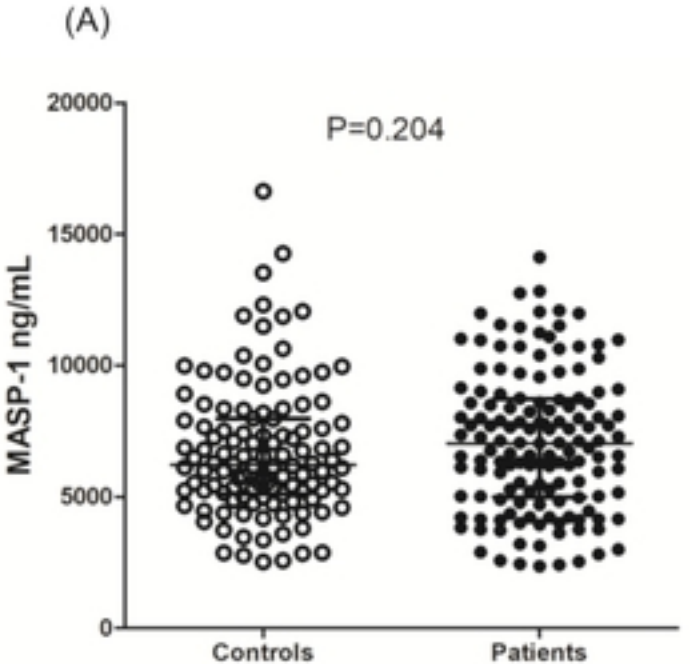
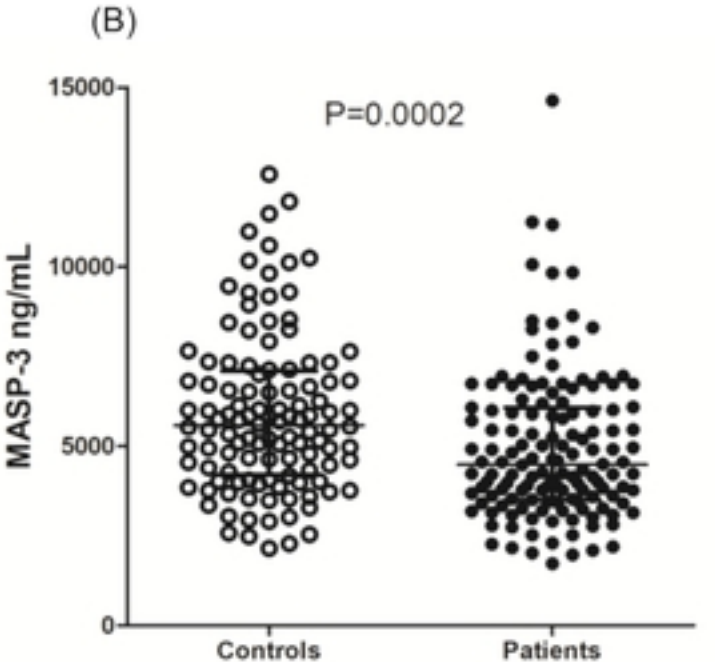

(A)
P=0.204
MASP-1 ng/mL
20000
15000
10000
5000
0
Controls
Patients


(B)
P=0.0002
MASP-3 ng/mL
15000
10000
5000
0
Controls
Patients

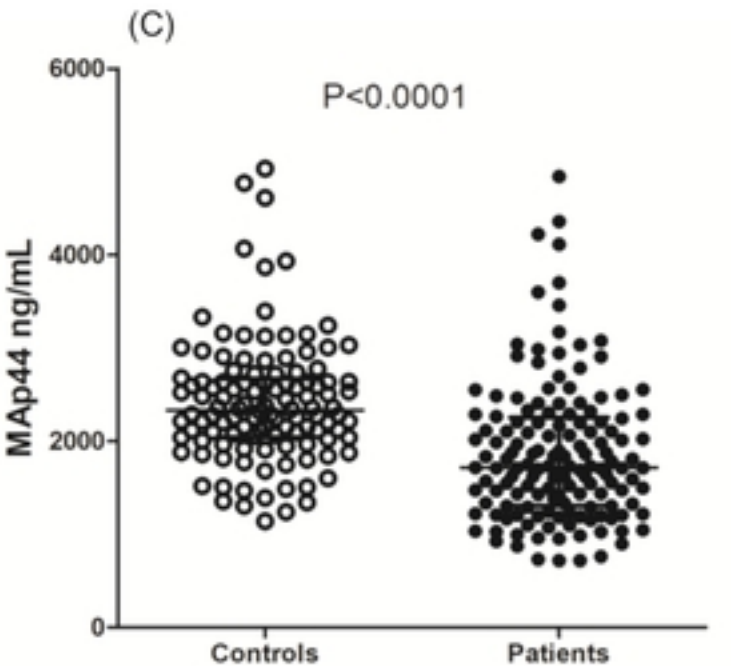

(C)
P<0.0001
MAp44 ng/mL
6000
4000
2000
0
Controls
Patients

Figure1

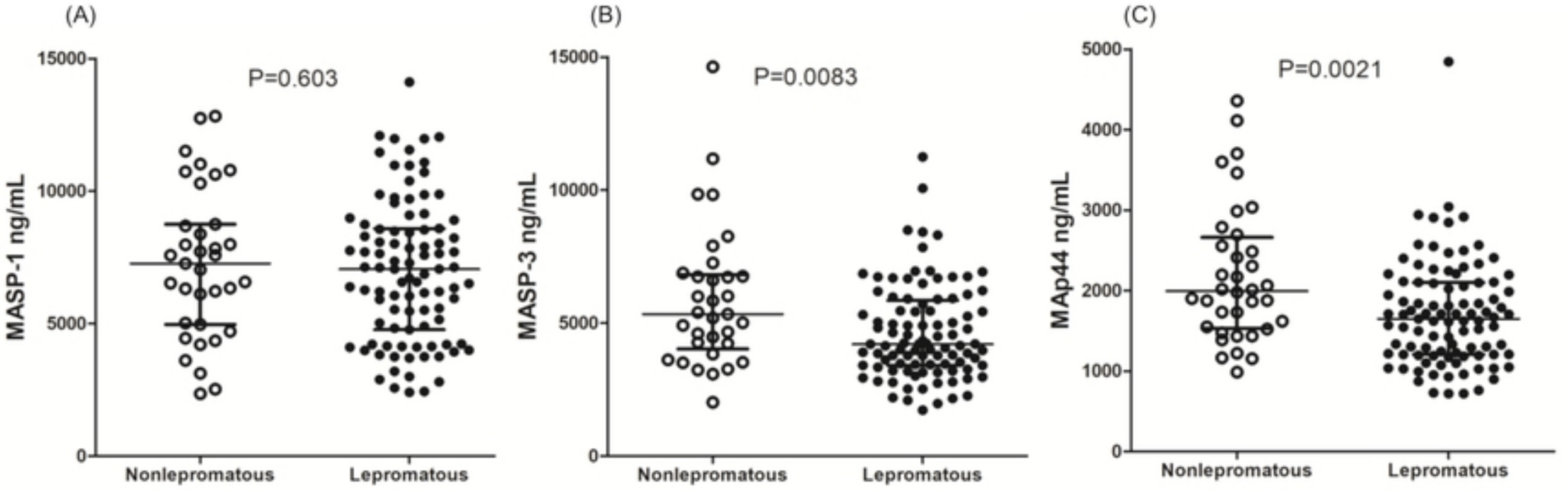

(A)
P=0.603
MASP-1 ng/mL
15000
10000
5000
0
Nonlepromatous
Lepromatous
(B)
P=0.0083
MASP-3 ng/mL
15000
10000
5000
0
Nonlepromatous
Lepromatous
(C)
P=0.0021
MAp44 ng/mL
5000
4000
3000
2000
1000
0
Nonlepromatous
Lepromatous

Figure2

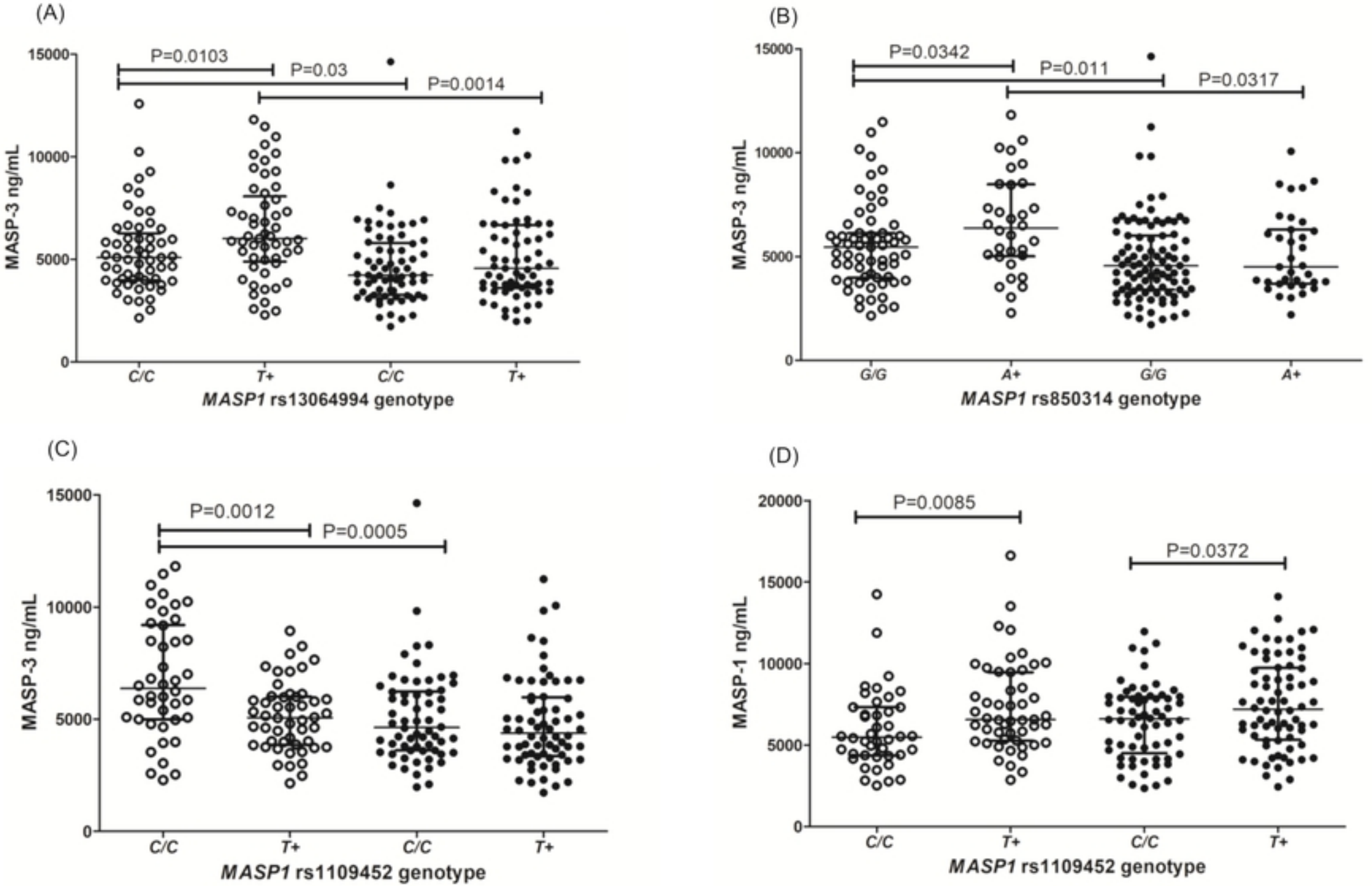
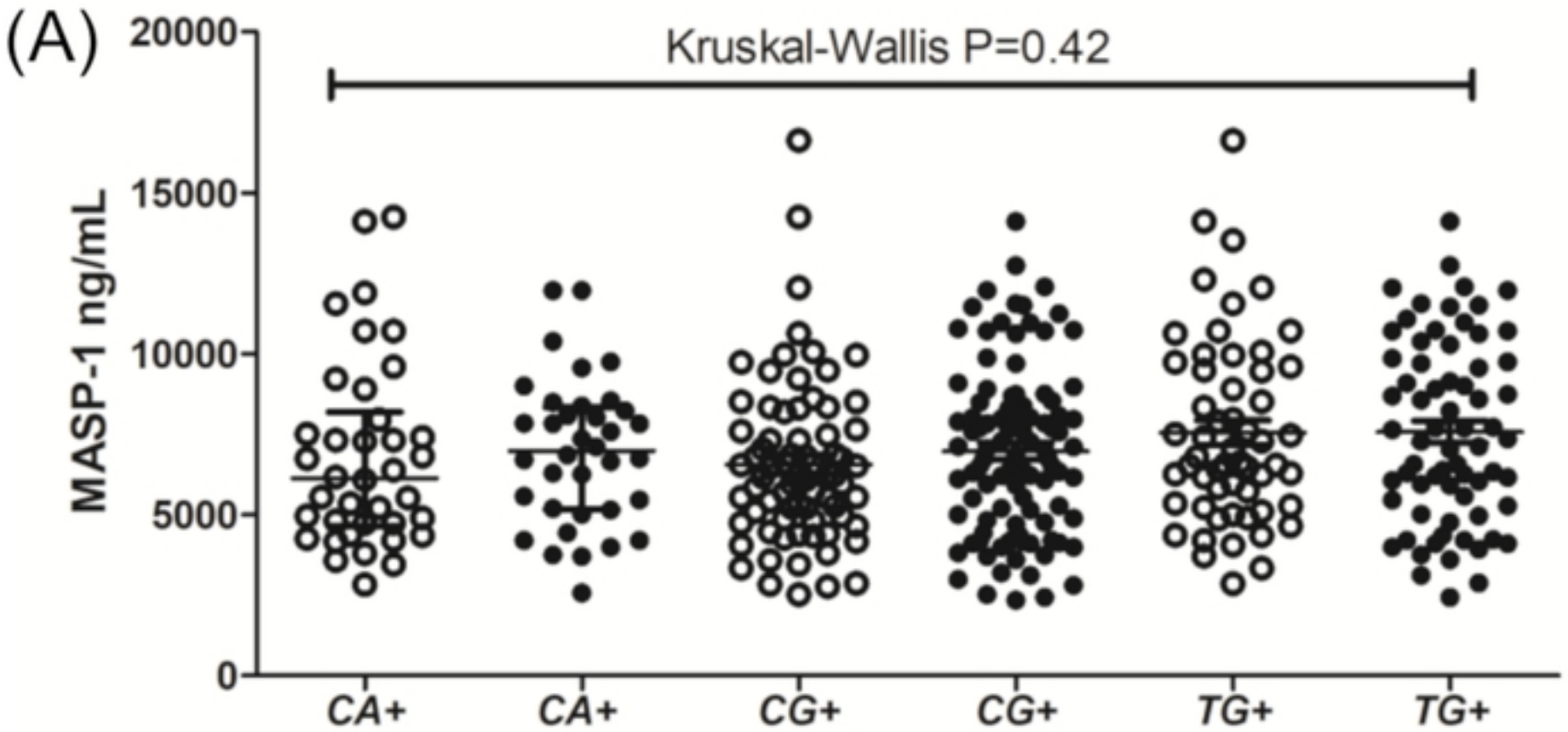
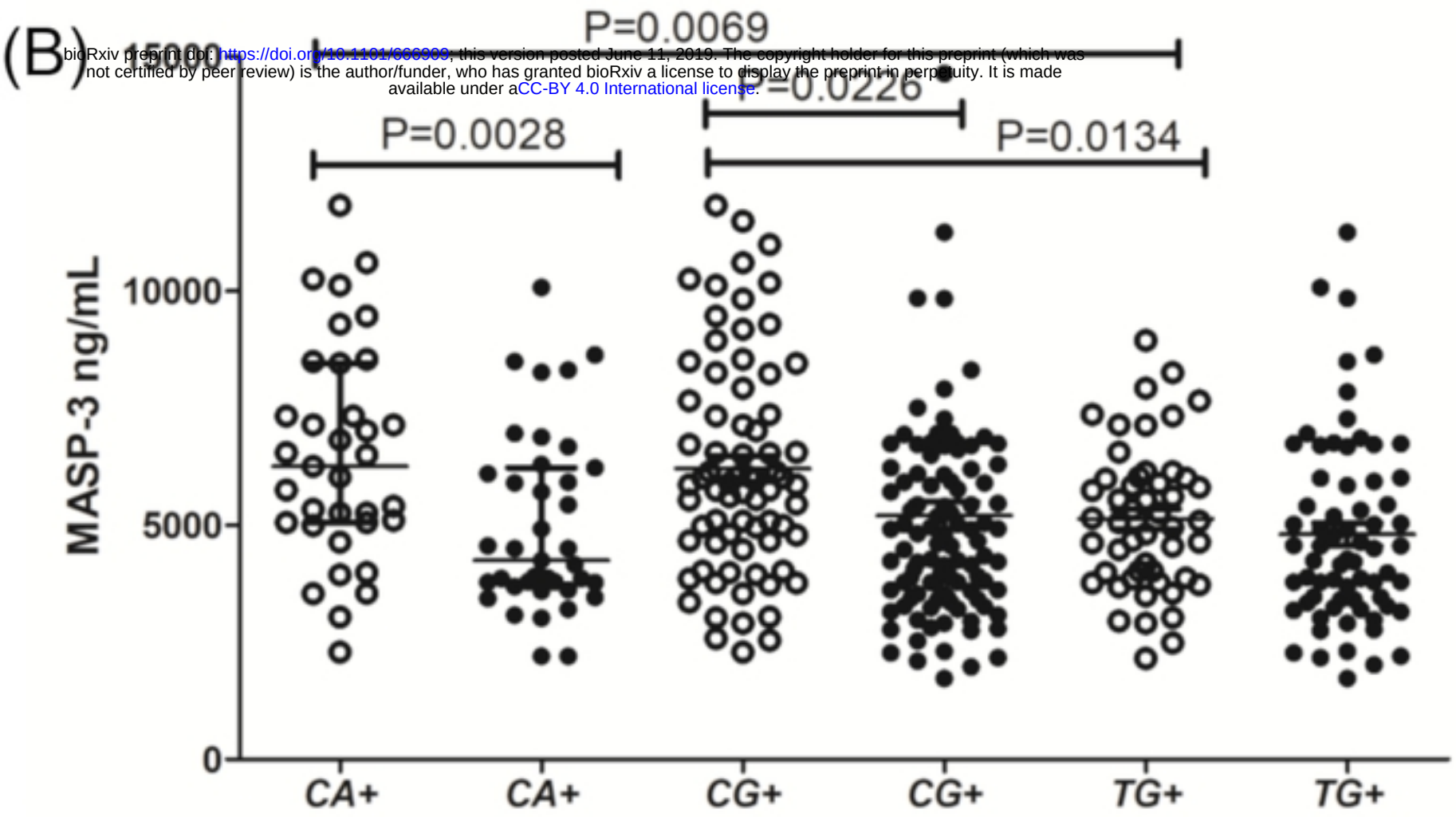

(A)
P=0.0103
P=0.03
P=0.0014
MASP-3 ng/mL
15000
10000
5000
0
C/C
T+
C/C
T+
MASP1 rs13064994 genotype
(B)
P=0.0342
P=0.011
P=0.0317
MASP-3 ng/mL
G/G
A+
G/G
A+
MASP1 rs850314 genotype
(C)
P=0.0012
P=0.0005
MASP-3 ng/mL
C/C
T+
C/C
T+
MASP1 rs1109452 genotype
(D)
P=0.0085
P=0.0372
MASP-1 ng/mL
20000
15000
10000
5000
0
C/C
T+
C/C
T+
MASP1 rs1109452 genotype

Figure3





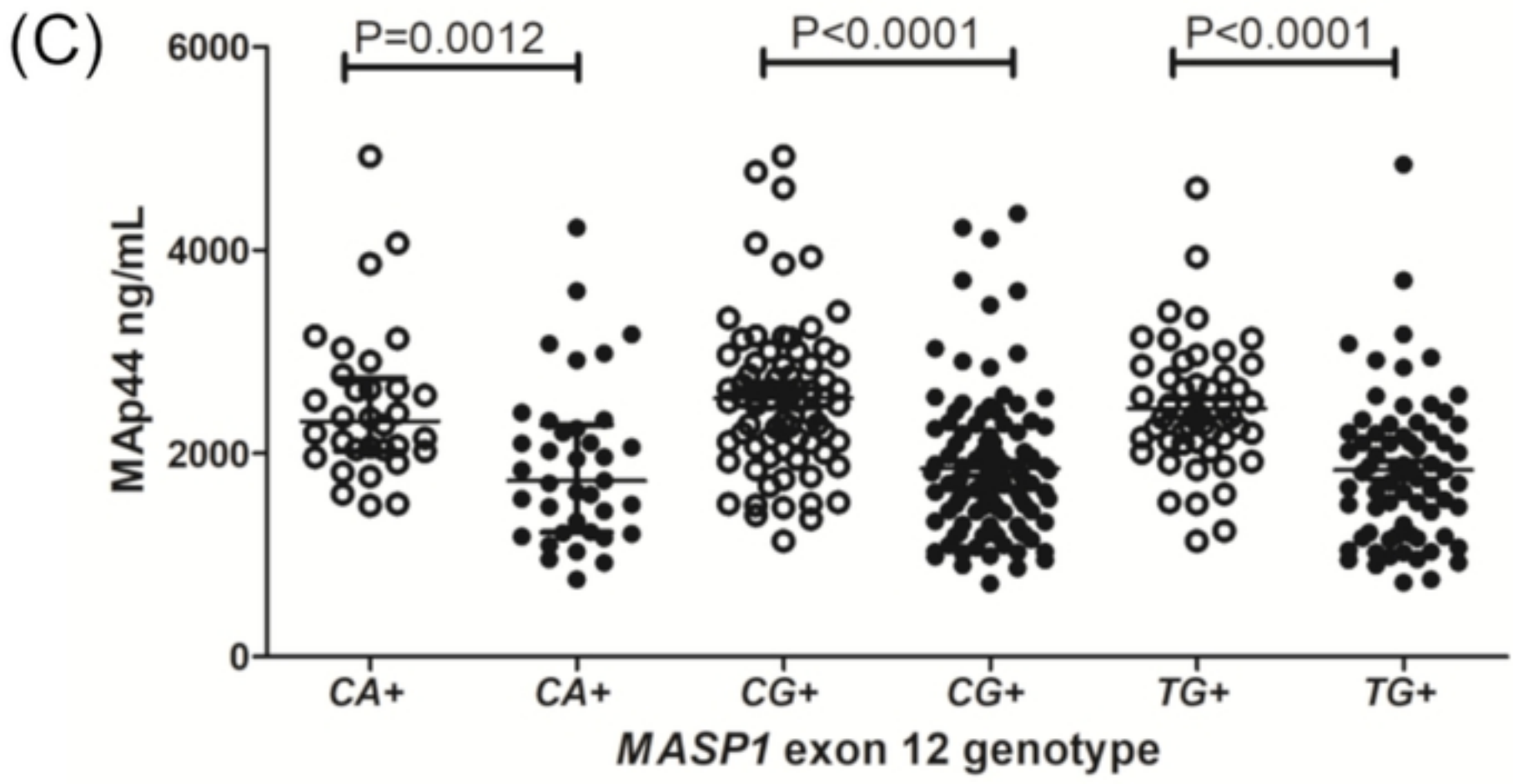


Figure4

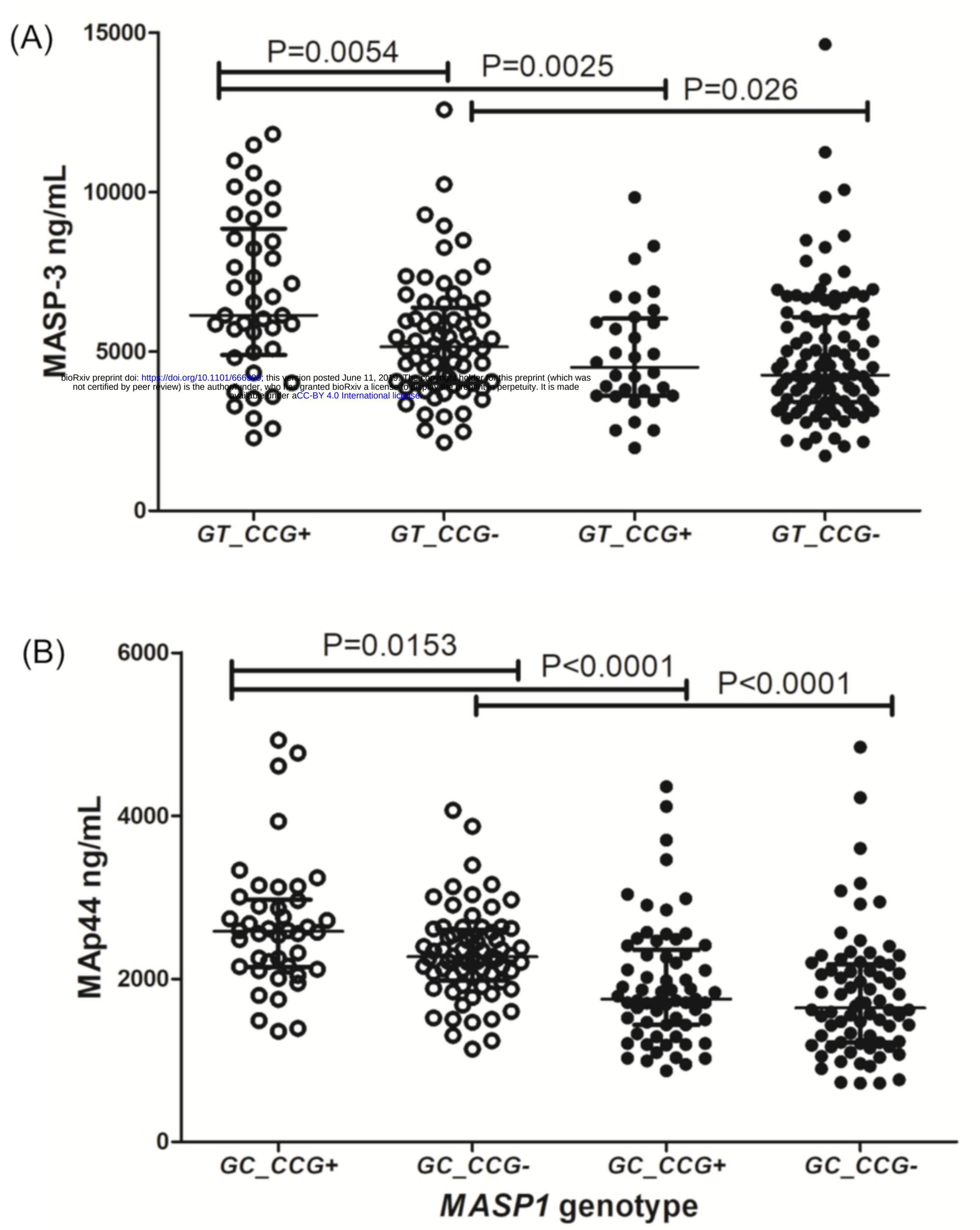


Figure5

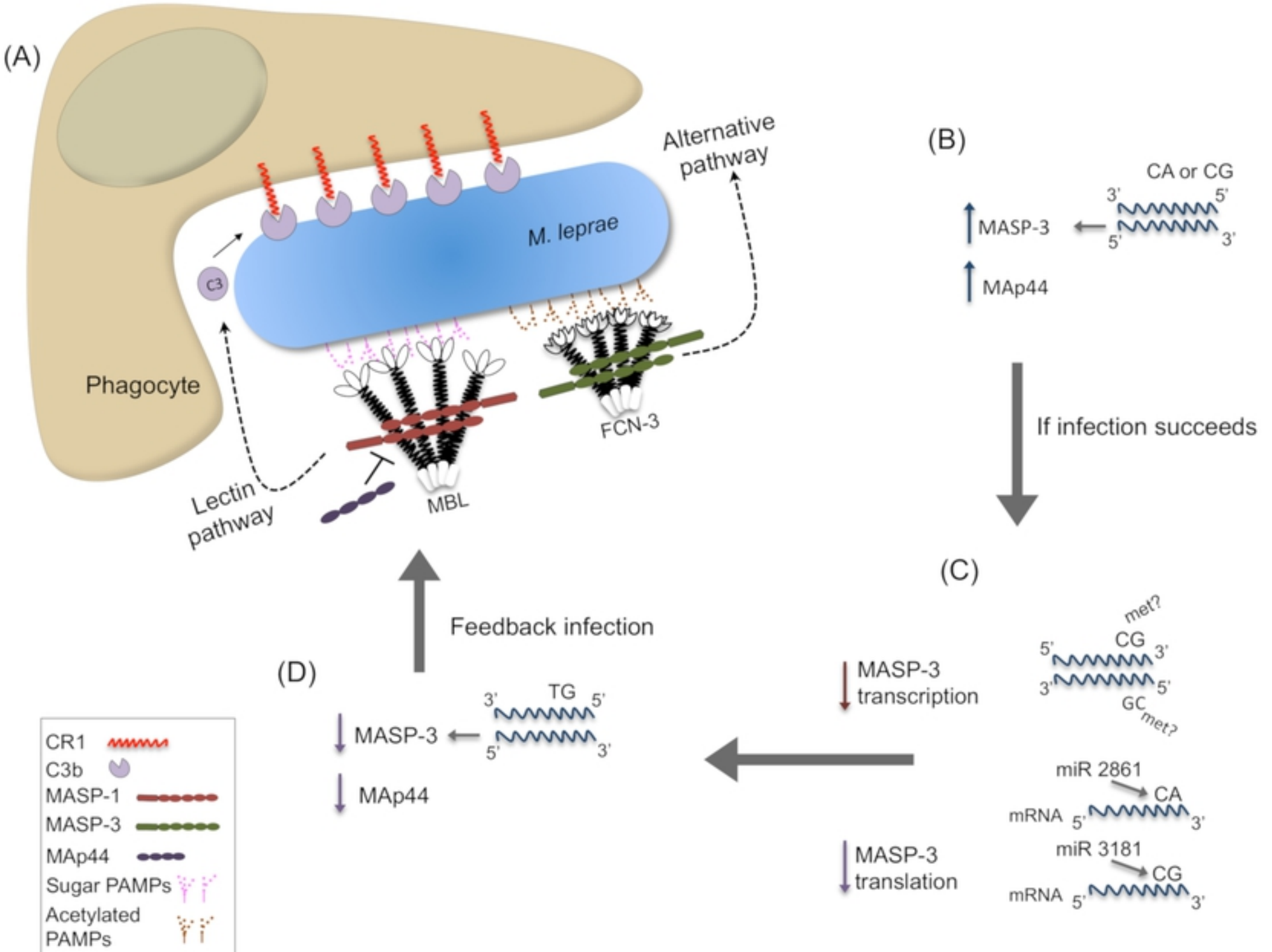

(A)
Phagocyte
M. leprae
C3
Alternative pathway
Lectin pathway
MBL
FCN-3
(B)
CA or CG
MASP-3
MAp44
If infection succeeds
(C)
met?
CG
GC
met?
miR 2861
CA
mRNA
miR 3181
CG
mRNA
MASP-3 transcription
MASP-3 translation
(D)
TG
MASP-3
MAp44
Feedback infection
CR1
C3b
MASP-1
MASP-3
MAp44
Sugar PAMPs
Acetylated PAMPs

Figure6