



# Mannose-binding lectin gene 2 variant DD (rs 5030737) is associated with susceptibility to COVID-19 infection in the urban population of Patna City (India)

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## Abstract

The study aimed to measure plasma levels of Mannose-Binding Lectin (MBL) and MBL-associated serine protease-2 (MASP-2) and their polymorphisms in COVID-19 patients and controls to detect association. As MBL is a protein of immunological importance, it may contribute to the first-line host defence against SARS-CoV-2. MBL initiates the lectin pathway of complement activation with help of MASP-1 and MASP-2. Hence, appropriate serum levels of MBL and MASPs are crucial in getting protection from the disease. The polymorphisms of MBL and MASP genes affect their plasma levels, impacting their protective function and thus may manifest susceptibility, extreme variability in the clinical symptoms and progression of COVID-19 disease. The present study was conducted to find plasma levels and genetic variations in MBL and MASP-2 in COVID-19 patients and controls using PCR–RFLP and ELISA, respectively. The present study was conducted to find plasma levels and genetic variations in MBL and MASP-2 in COVID-19 patients and controls using PCR–RFLP and ELISA, respectively. Our results indicate that median serum levels of MBL and MASP-2 were significantly low in diseased cases but attained normal levels on recovery. Only genotype DD was found to be associated with COVID-19 cases in the urban population of Patna city.

**Keywords** MBL genotyping · MASP-2 · SARS-COV-2 · COVID-19

## Introduction

Coronavirus Disease 2019 (COVID-19) emerged as a public health emergency with high morbidity and extreme variability in the clinical presentation (Aminjafari et al. 2020). The

disease has been extensively investigated from almost all aspects. At the molecular level, it is pertinent to explore the role of immune proteins in this disease which remained hitherto uninvestigated. The levels of some important immune proteins and their possible genetic variants may contribute to the pathogenesis of COVID-19. Indeed, severe SARS-CoV infections have often been observed to have an underlying immune-mediated pathology driven by the complement activation (Darnell et al. 2007). Mannose-Binding Lectin (MBL) is a protein of immunological importance and may have a significant contribution in the first-line host defence against SARS-CoV-2 (Lardone et al. 2021; Matricardi et al. 2020). It binds to the mannan and N-acetylglucosamine residues on viral or virus-infected cellular surfaces and initiates the lectin pathway (LP) of complement activation. This leads to the auto-activation of MBL-associated serine protease-1 (MASP-1), which in turn activates zymogen MASP-2 which follows the activation of LP (Héja et al. 2012). Hence, their levels are crucial to get protection from the disease which is governed by the expression of their genes.

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MBL often has three structural and two promoter gene polymorphisms that are responsible for its low serum levels (Eisen and Minchinton 2003). These are single base changes in codons 52, 54 and 57 of the exon leading to Arg-52 to Cys (D variant), Gly-54 to Asp (B variant) and Gly-57 to Glu (C variant) conversions (Garred et al. 2006). The nucleotide substitutions at positions –550, –221 and +4 in the promoter region result in H/L, Y/X and P/Q variants of MBL, respectively. Haplotypes HY and LY are associated with high plasma MBL levels whereas LX shows low levels (Garred et al. 2006; Ammitzbøll et al. 2013). A reduced MBL expression caused by genetic polymorphisms increases the susceptibility to SARS-CoV infection (Zhang et al. 2005).

In MASP-2, two SNPs (single nucleotide polymorphisms) namely p.D120G and p.R99 located in exon 3 of the gene are important and account for its deficiency (Knudson 2001; Goeldner et al. 2014). The p.D120G SNP determines MASP-2 deficiency in approximately 0.3% of European individuals (Thiel et al. 2007; Stengaard-Pedersen et al. 2003).

LP activation plays a key role in the immunopathogenesis of COVID-19 (Ali et al. 2021). Here direct binding of MASP-2 to the N protein of SARS-CoV-2 with subsequent activation of LP was demonstrated in vitro. This study further supported the findings of Gao et al. who have also reported direct activation of MASP-2 on the SARS-CoV-2 N protein and showed that MASP-2-deficient mice were protected from the disease (Gao et al. 2020).

MASP-2, an LP effector enzyme, has also been found to be deposited significantly in the lung and/or skin of patients with severe COVID-19, which is attributed to an over-activated complement system (Magro et al. 2020). These findings indicate the vital role of MBL and MASP-2 in COVID-19 infection, hence their plasma levels are critical in protecting from the disease.

The polymorphism of MBL and MASPs genes affects their plasma levels, impacting their protective function and thus may manifest extreme variability in the symptoms. In view of the above, a study was conducted to find out genetic variations in MBL and MASP-2 genes in COVID-19 patients. The plasma levels of MBL and MASP-2 were also evaluated during and after recovery from the COVID-19 infection. To the best of our knowledge no such report on levels of these proteins, before, during and after recovery from COVID-19 infection has been published to date.

## Methodology

A total of 100 cases and 100 control subjects were recruited for this study. The cases were patients who tested RT-PCR positive for SARS-CoV-2 (henceforth termed as RTPP) and were admitted to the hospital. All cases were stable and mildly symptomatic. The comorbidities were not recorded in these patients. The cycle threshold (ct) value for the positivity of infection in the PCR was <35 for all samples. The controls were RT-PCR negative (henceforth termed RTPN) and did not have any flu-like symptoms. This study was approved by the Ethical Committee of All India Institute of Medical Sciences Patna (IEC) (Ref. No. AIIMS/Pat/IEC/2020/528). All the participants gave informed consent for their blood samples.

Following institute guidelines, the employees of the institute who were symptomatic or came in contact with confirmed positive COVID-19 cases were tested by RT-PCR. The institutional database of these individuals was utilized by the research group for sample collection. The study was conducted between the period of October–December 2020.

Ethylene diamine tetra-acetic acid (EDTA) vacutainers were used for collecting the test samples. They were centrifuged at 3000 rpm for 10 min within 1 h after venipuncture, and the plasma was stored at –20 °C for further analysis. Plasma levels of MBL and MASP-2 were evaluated in the cases and controls group and also when the cases recovered from the disease and tested RT-PCR negative (henceforth termed as recovered cases). The cells from the blood samples were used for the isolation of DNA for genotyping of MBL and MASP-2.

Forty plasma samples, each from cases, controls and recovered cases were investigated for evaluation of levels of MBL and MASP-2 by ELISA kits (Bioassay Technology, Shanghai Korain Biotech Co) following the manufacturer's protocol with slight modifications. The recovered cases were the same patients who recovered from the infection and their blood samples were taken only after these cases tested negative by RT-PCR. Plasma sample (40 µl), biotinylated human antibody (10 µl), and streptavidin Horseradish Peroxidase (HRP) (50 µl) were added to the ELISA plate with pre-coated specific antibodies. The plate was incubated for 1 h at 37 °C, then washed using 1X washing buffer on an automated ELISA washer (SW40, Bio-Rad California, USA). Afterwards, 50 µl each of substrate solutions A and B was added and incubated for 10 min at 37 °C. Hydrogen peroxide (H<sub>2</sub>O<sub>2</sub>) solution (50 µl) was added and reading was taken within 10 min at 450 nm on a microplate reader (PR4100 Bio-Rad USA) and calculated with curve-fitting software (Magellan, PR4100 Bio-Rad, USA). Genomic DNA from 100 cases

**Table 1** Primer sequences used for *MBL* and *MASP-2* gene analysis

S. no	MBL primers			Annealing temperature	Product size (bp)
1	Promoter	<i>Forward</i>	gaaaatgcttaccaggacaagcctgt	61 °C	134
		<i>Reverse</i>	gtctctcatatccccaggc		
2	Exon	<i>Forward</i>	catcaacggctcccaggcgacaagatggg	62 °C	410
		<i>Reverse</i>	gtctctcatatccccaggc		
<i>MASP-2 Primers</i>					
3	rs61735600	<i>Forward</i>	agagcacagacacggagca	59 °C	118
		<i>Reverse</i>	gcctcgaaccccgtaaca		
4	rs72550870	<i>Forward</i>	caggtcactggacaacagatca	57 °C	695
		<i>Reverse</i>	gcttctcgttgagtagc		

and controls was extracted using a DNA isolation kit (Wizard total DNA isolation kit, Promega) following the manufacturer's protocols and the DNA concentration was measured using Nano-drop (Thermo-scientific).

Polymerase chain reaction–restriction fragment length polymorphism (PCR–RFLP) was used to detect the MBL genotypes (Boldt et al. 2011). In brief, PCR was performed in a 20 µl reaction containing 1 × PCR buffer, 1.5 mM MgCl<sub>2</sub>, 0.5 mM each dNTP, specific sets of primers (Table 1), 100 ng DNA, 0.5U Taq DNA polymerase (New England BioLabs, Hitchin, UK). The conditions for amplification were as follows: Initial denaturation step of 8 min at 95 °C, followed by 35 cycles of 30 s denaturation at 95 °C, 30 s annealing at 60 °C, 45 s extension at 72 °C, and a final elongation step of 5 min at 72 °C. Amplicons were accurately sized by agarose gel electrophoresis. Restriction digestion reaction was performed according to the manufacturer's instruction (New England BioLabs, Hitchin, UK). For MBL exon1 PCR products were digested by using MwoI (cleaves codon 52), BanI (cleaves codon 54), MboII (cleaves codon 57); For MBL Promoter DrdI (cleaves L allele at 550) and BtgI (cleaves Y alleles at 221). Subsequently, PCR products were incubated at 37 °C for 15 min; then the reaction was stopped at 65 °C for 5 min. Finally, SNPs screening was achieved by performing agarose gel electrophoresis and bands were visualized under a UV transilluminator. The genotypes of the *MASP-2* gene were detected by using Multiplex PCR with sequence-specific primers (PCR-SSP) modified from Boldt et al. (Boldt et al. 2011). PCR was carried out with 100 ng DNA, 0.2 mM dNTP (Invitrogen, São Paulo, Brazil), 1 × PCR buffer (Qiagen), and 0.5U/µL Taq polymerase (Invitrogen) in a final volume of 20µL in an Eppendorf Mastercycler Nexus Gradient Thermal Cycler (Eppendorf, USA). The conditions for amplification were as follows: Initial denaturation step of 07 min at 95 °C, followed by 35cycles of 30 s denaturation at 95 °C, 30 s

annealing at 58 °C, 30 s extension at 72 °C, and a final elongation step of 5 min at 72 °C. The annealing temperature was decreased every 5cycles, according to the previous “touch-down” strategy with some minor modifications. Finally, amplified products were electrophoresed, and 6µL of PCR product was loaded into a 2% (w/v) agarose gel in 1 × TBE. The gels were run for 1 h at 100 V, and photographs were taken under a UV transilluminator. Thereafter, the presence and absence of specific bands were used to identify *MASP-2* variants and haplotypes.

An R-package “SNPassoc” was used for the analysis of genome association studies and calculation of Hardy–Weinberg equilibrium. The analysis of association was based on generalized linear or regression models (González et al. 2007). Since the collected data was not distributed uniformly, the non-parametric Wilcoxon test was used for analysis. Significance was accepted as  $p < 0.05$  for the results of all analyses.

## Results and discussion

The genotyping was carried out on a total of 200 subjects, 100 each in the control and patients group. Table 2 shows the detailed information of the participants. Forty samples from patients and controls were investigated for plasma levels of MBL and *MASP-2* (Fig. 1A & B). These levels were also evaluated in the samples of the same patients after recovery from the disease (recovered cases). It may be noted that as we could get only forty patients' serum samples after recovery, therefore we included these samples for evaluation of levels of MBL and *MASP-2*. We used multivariate analysis to establish a relationship between disease occurrence with the plasma levels and genetic variants of MBL and *MASP-2* among the cases and controls. Gender is adjusted as an explanatory variable in the regression.

**Table 2** Study participants details

Study participants detail	Cases (RT PCR +ve) ( <i>n</i> = 40)	Controls (RT PCR –ve, without symptoms) ( <i>n</i> = 40)
Age, mean (95% CI)	54.25 (49.8–59.1)	27.25 (26.19–28.30)
Male, no. (%)	32 (80)	23 (57.5)
Female, no. (%)	08 (20)	17 (42.5)
Fever (38° C), no. (%)	40 (100)	None of the participants had any symptom in this group
Cough, no. (%)	40 (100)	
Sore throat, no. (%)	32 (80)	
Difficulty in breathing, no. (%)	32 (80)	
Tiredness, no. (%)	40 (100)	
Chest pain, no. (%)	29 (72.5)	
Breathlessness, no. (%)	26 (65)	
Loss of taste, no. (%)	23 (57.5)	
Loss of smell, no. (%)	19 (47.5)	
Body ache, no. (%)	38 (95)	
Headache, no. (%)	32 (80)	

\*Population: Asian Indian population from the Eastern Indian state of Patna

Table 3 shows the distribution of studied genotypes among the subjects. Table 4 summarizes the disease prevalence association of the different identified genotypes. The prevalence of H/H genotype numbers was significantly lower in COVID-19 cases when compared to controls (30% vs 46%, respectively; OR = 4.0,  $p < 0.0001$ ). However, the prevalence of H/L and L/L genotypes was non-significant in between the COVID-19 cases and controls. Another promoter site (–221) X/Y genotypes were also found to be not associated with the prevalence of the disease. The disease probability was also calculated by counting the gene/mutation when found in combination with the interacting gene/mutation (Table 5). The co-presence of alleles was found to have no direct correlation ( $p > 0.05$ ). In the case of MBL2 exon genotypes, the D allele was found to be associated with a higher prevalence of COVID-19 (Table 4). Individuals with D-type mutation were more prone to the disease (17% vs 5% in case and control respectively;  $p < 0.0008$  at 95% of confidence intervals. AD genotype numbers in COVID-19 and control are both six, the result is not statistically significant. This gene may play a role in the pathogenesis of SARS-CoV-2 in the urban population of Patna city, which is the capital city of the state of Bihar in India.

The median serum levels of MBL and MASP-2 were found to be significantly lower in the diseased cases (RTPP) compared to controls (RTPN) (Fig. 1A & B). The MBL level was 418 ug/L in disease cases as compared to 2249ug/L in normal ( $P < 0.001$ ). The MASP-2 level in diseases cases was 242 ug/l as compared to 668 ug/L in control ( $p = 0.049$ ). After the recovery of the disease MBL and MASP-2 levels

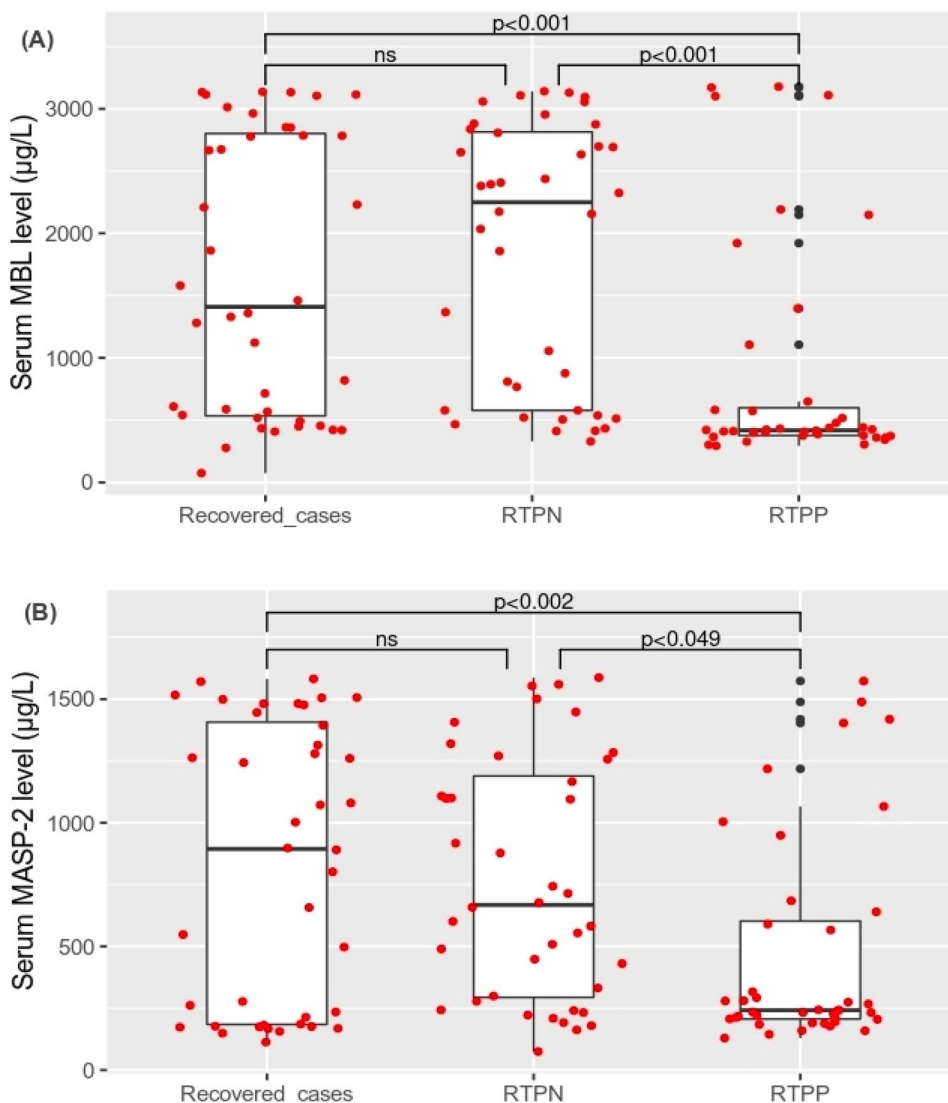
attained normal levels again (MBL 1410 ug/l and MASP-2 894 ug/l in recovered cases respectively) (Fig. 1A & B).

Studies on individual genotypes for MBL and MASP-2 in cases and controls were also analysed to understand if any relationship exists with COVID-19. All genotypes viz MBL exons, promoters and MASP-2 exons (apart from DD) show no specific association with the disease (Fig. 2A & B). Plasma MBL levels were found to be reduced in disease conditions despite no significant genotype variations. Likewise, in both promoter genotypes, plasma MBL levels were found to be reduced in case samples. In a limited number of severe SARS-CoV-2 patients in our study (only 4), a genetic association between MBL polymorphism and intensive care unit (ICU) admission or death due to SARS-CoV-2 could not be determined.

MBL contributes significantly to innate immunity. MBL responses are highly heterogeneous. In the acute phase of the disease, the heterogeneity in protein responses is possibly influenced by the genetic differences in both structural and regulatory parts of the gene (Herpers et al. 2009). Its concentration is dependent on various mutations present both in the exon and promoter region of MBL (Garred et al. 2006; Zhang et al. 2005; Ip et al. 2004). Similarly, concentrations of associated proteases, MASPs, are also governed by mutations in the genes. Thus, mutations affecting the concentrations of MBL or MASPs may confer susceptibility to certain infections (Ip et al. 2004; Kalia et al. 2021; Tao et al. 2012; Takahashi et al. 2005).

Our study conducted on an urban population of Patna city found only one type of genetic variant of MBL i.e. D type, which showed association with the disease and otherwise no genetic variants or mutations in MBL or MASP-2 were

**Fig. 1** **A** Serum levels for MBL for RTPN (RTPCR negative), RTPP (RTPCR positive) and recovered cases. The horizontal bar indicates the median for each group. **B** Serum levels for MASP-2 for RTPN (RTPCR negative), RTPP (RTPCR positive) and recovered cases. The horizontal bar indicates the median for each group. The black and red dots represent the outliers and an individual’s serum MASP-2/MBL levels respectively



**Table 3** Distribution of studied genotypes and allele frequencies in MBL

Promoter	Genotype frequencies			Allele frequencies	
	XX	XY	YY	X allele	Y allele
HWE: > 0.001	92	48	56	0.552	0.447
Exon	AA	AD	DD	Wild type	D allele
HWE: > 0.001	112	12	22	0.808	0.191
	AA	AC	CC	Wild type	C-allele
HWE: > 0.001	112	4	24	0.814	0.185
	AA	AB	BB	Wild type	B allele
HWE: > 0.001	112	5	21	0.829	0.1703

HWE Hardy–Weinberg Equilibrium

found to be associated with COVID-19 in our study

participants. However, other studies on SARS-CoV done earlier reported an association of BB allele (Zhang et al. 2005).

A study from Turkey reported B variants of the MBL2 gene associated with lower levels and related to a higher risk for a more severe clinical course of COVID-19 infection in some respects (Medetalibeyoglu et al. 2021).

In a study of 4 independent case–control populations of Chinese descent including 932 patients and 982 controls, the B allele (rs1800450) of MBL and GG genotype (rs 1,024,611) at CC12 (chemokine ligand 2) were found to be associated with increased risk of SARS-CoV infection. The SNPs of CCL2 and MBL increase the susceptibility to SARS-CoV infection in a cumulative manner. (Ramanaathan et al. 2020). COVID-19 patients and 392 controls a statistically significant association was obtained in susceptibility to SARS-CoV infection and having B allele (Zhang

**Table 4** Disease association of the mutations

Genotype	Case (n, %)	Control (n, %)	Crude OR (95 CI%)	<i>p</i> *
<b>Promoter (–221)</b>				
X/X	71	82	1.0	0.205
X/Y	13	8		
Y/Y	16	10	0.58	
<b>Promoter (–550)</b>				
H/H	60	91	4.0	<0.0001*
H/L	25	4		
L/L	15	5	0.30	
<b>Exons</b>				
AA	43	69	1.0	
BB	12	09	0.47	0.141
AB	04	01	0.16	
CC	14	10	0.45	0.073
AC	04	00	0	
DD	17	05	0.18	0.0008*
AD	06	06	0.62	

OR Odds ratio, CI confidence intervals, *p*\*: by Chi-square test. \*Disease association with allele. Gender is adjusted as an explanatory variable in the regression

**Table 5** Inter-mutation interactions and disease probability study

Gene/mutation	Interacting gene/mutation	<i>p</i>
Promoter (–221)	Exon 54 (B)	0.99
	Exon 57 (C)	0.47
	Exon 52 (D)	0.17
	Promoter (–550)	0.41
Promoter (–550)	Exon 54 (B)	0.24
	Exon 57 (C)	0.37
	Exon 52 (D)	0.24

et al. 2005). No significant association between alleles or genotype of MASP-2 and susceptibility to SARS-CoV was observed in the Chinese population (Wang et al. 2009).

Although we did not find remarkable genetic variations among cases, our results on the serum levels of MBL and MASP-2 were strikingly unusual. Their levels were found significantly low in the diseased cases compared to controls and interestingly these levels reached normal after recovery from the disease. We speculate that in our study of mild to moderate cases, MBL was fully consumed to fight the COVID-19 infection and thus the levels were reduced. It also implies that in our study the levels were

not governed by variant genotypes and recovered on recovery from infection with SARS-CoV-2. MBL levels after recovery from the disease were not measured as done in this study.

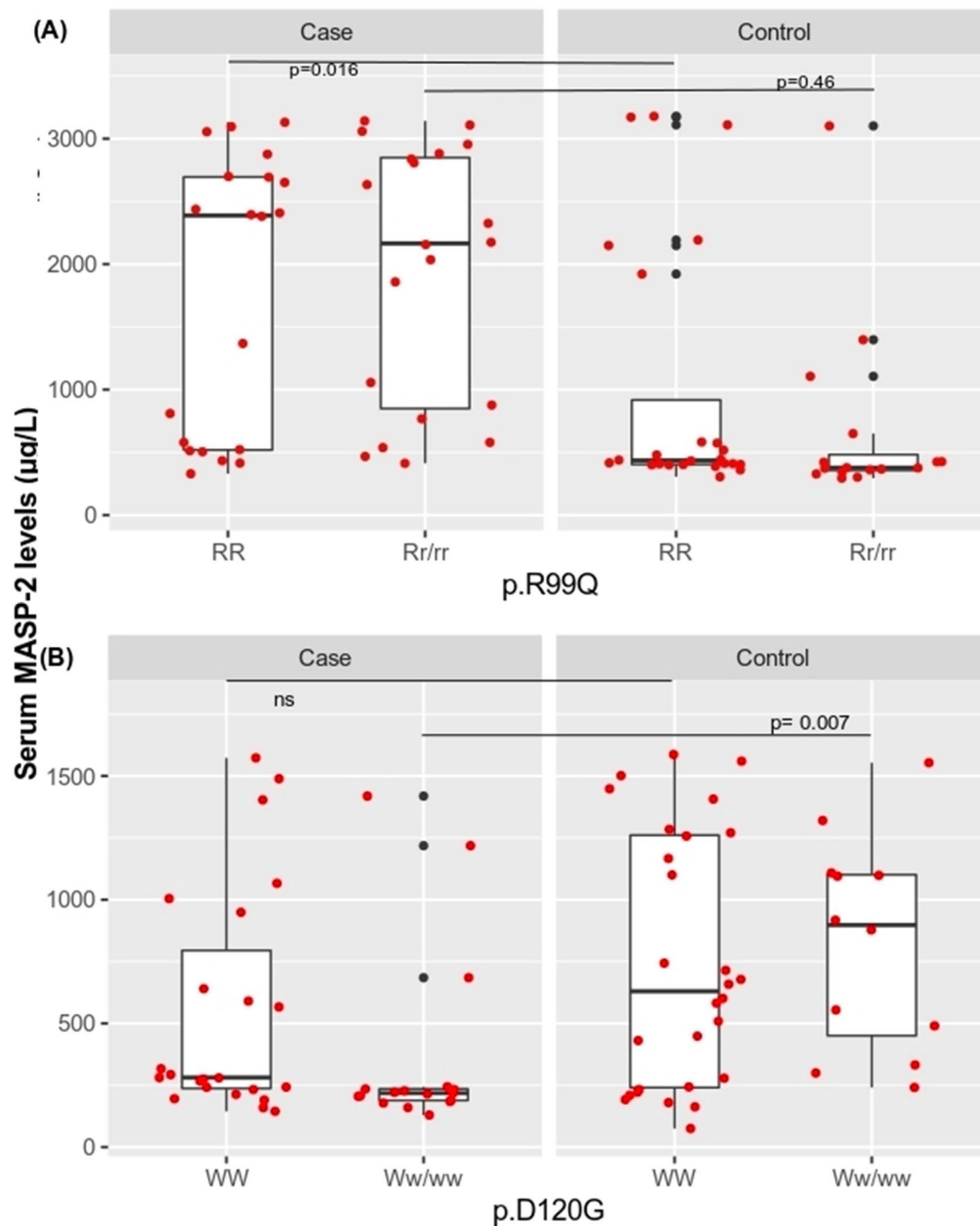
A study has already reported that low levels of MBL help in eliminating the hepatitis C virus (Zhang et al. 2020). In contrast, higher serum MBL levels positively correlate to the mortality risk in ICU patients with A(H1N1) pdm09 infection (Zogheib et al. 2018).

Although there are several reports that a decreased MBL expression will lead to a decreased innate immunity and risk of SARS-CoV-2 infection (Glotov et al. 2021, Ip 2005, Larsen et al. 2004), and that MBL and MASP-2 deficiencies derived from polymorphisms may be responsible for disease susceptibility, in COVID-19 infection, low levels of MBL and MASP-2, independent of polymorphism, might have been the result of their utilization fighting the infection. Such low levels of MBL and MASP-2 might have further helped in the clearance of viral infection as has been reported in the case of HCV infection (Tulio et al. 2011). On the same lines, another study by Silva et al. (2018) has reported that MASP-2 levels were decreased in chronic disease independently of MASP-2 genotypes. This phenomenon may be caused by consumption and attenuation mechanisms of viral origin and a reduced liver function, the latter being responsible for the production of MASP-2 (Silva et al. 2018).

It is important to note that the majority of the studies have been done on acute/severe cases of COVID-19 infection and have reported higher levels of MBL and over-activated complement system of LP in acute infection leading to tissue damage and coagulopathy (Magro et al. 2020; Eriksson et al. 2020), however, we took mild to moderate patients of COVID-19 and evaluated levels of MBL and MASP-2 levels, which we repeated after the recovery from the disease. To the best of our knowledge, we did not come across a study where levels were measured after recovery from the disease.

## Conclusion

The low levels of MBL and MASP-2 in mild to moderate patients of COVID-19 were found to be independent of their genotypes. Since the levels of MBL and MASP-2 recovered once the infection subsided, it rules out the possibility of polymorphism leading to low levels of these proteins in COVID-19 infection. Clinical symptoms of COVID-19 infection manifested with low levels of MBL and MASP-2. Only genotype DD was found to be



**Fig. 2** **A** Serum levels of MASP-2 for wild-type WW and variants Ww/ww alleles for p.R99Q in COVID-19 cases and controls. The horizontal bar indicates the median for each genotype subgroup. **B** Serum levels of MASP-2 for wild-type WW and variants Ww/ww

alleles for p.D120G in COVID-19 cases and controls. The horizontal bar indicates the median for each genotype subgroup. The black and red dots represent the outliers and an individual's serum MASP-2/MBL levels respectively

associated with COVID-19 cases in the urban population of Patna city.

### Limitation of the study

The sample size of the study participants should have been large and also serum MBL and MASP-2 levels should have

been measured in all the patients.

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## Declarations

**Conflict of interest** All authors have no competing interests to declare.

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