

# MBL2 Variations and Malaria Susceptibility in Indian Populations

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**Human mannose-binding lectin (MBL) encoded by the *MBL2* gene is a pattern recognition protein and has been associated with many infectious diseases, including malaria. We sought to investigate the contribution of functional *MBL2* gene variations to *Plasmodium falciparum* malaria in well-defined cases and in matched controls. We resequenced the 8.7 kb of the entire *MBL2* gene in 434 individuals clinically classified with malaria from regions of India where malaria is endemic. The study cohort included 176 patients with severe malaria, 101 patients with mild malaria, and 157 ethnically matched asymptomatic individuals. In addition, 830 individuals from 32 socially, linguistically, and geographically diverse endogamous populations of India were investigated for the distribution of functional *MBL2* variants. The *MBL2* –221C (X) allelic variant is associated with increased risk of malaria (mild malaria odds ratio [OR] = 1.9, corrected *P* value [*P*<sup>Corr</sup>] = 0.0036; severe malaria OR = 1.6, *P*<sup>Corr</sup> = 0.02). The exon1 variants *MBL2*\*B (severe malaria OR = 2.1, *P*<sup>Corr</sup> = 0.036; mild versus severe malaria OR = 2.5, *P*<sup>Corr</sup> = 0.039) and *MBL2*\*C (mild versus severe malaria OR = 5.4, *P*<sup>Corr</sup> = 0.045) increased the odds of having malaria. The exon1 *MBL2*\*D/\*B/\*C variant increased the risk for severe malaria (OR = 3.4, *P*<sup>Corr</sup> = 0.00045). The frequencies of low MBL haplotypes were significantly higher in severe malaria (14.2%) compared to mild malaria (7.9%) and asymptomatic (3.8%). The *MBL2*\*LYPA haplotypes confer protection, whereas *MBL2*\*LXPA increases the malaria risk. Our findings in Indian populations demonstrate that *MBL2* functional variants are strongly associated with malaria and infection severity.**

Malaria is one of the major causes of morbidity and mortality in tropical and subtropical countries, with an estimated 1.24 million mortality in 2010, mostly in Africa (1, 2). Human malaria is caused by the hematozoan parasite of the genus *Plasmodium* with five different species (i.e., *P. falciparum*, *P. vivax*, *P. ovale*, *P. malariae*, and *P. knowlesi*) infecting humans. The pathogenesis of malaria is complex and multigenic, and the severity depends on the complex interplay between host genetic makeup, the parasite virulence, and its transmission dynamics, as well as the host immune responses (3, 4). Innate immune components mediate humoral immune responses to malaria by involving various complement proteins (5, 6). The innate immune system mainly relies on diverse pattern-recognizing receptor or soluble pathogen-recognizing molecules (PRMs) (7, 8) that recognize molecular motifs on the surfaces of microorganisms. Mannose-binding lectin (MBL) is a C-type soluble PRMs secreted by the liver as part of the acute-phase response and plays an important role in the recognition, initiation, regulation, and amplification of innate immune defense (9). The human MBL encoded by *MBL2* mapped to 10q21.1 is a 32-kDa polypeptide, made up of 248 amino acids, having an N-terminal cysteine-rich segment, a collagen-like domain, a helical coiled-coil neck region, and a C-terminal glucoconjugate recognition domain (10). The structural subunit is a homotrimer of 96 kDa with a triple helix at its collagen-like domain. The functional MBL complex is a bouquet-like hexamer that circulates in serum (11, 12). MBL binds to an array of glycoconjugates on the surface of bacteria, yeasts, viruses, and parasitic protozoa, including *P. falciparum* (13), and activates the complement system. MBL interacts with MBL-associated serine proteases (MASP-1, -2, and -3 and Map19) and activates the membrane attack complex or complement-mediated phagocytosis (9, 11, 13) and also regulates the release of proinflammatory cytokines (14).

*MBL2* genetic variants have been shown to associate with many infectious diseases such as malaria, leishmaniasis, leprosy, schistosomiasis, trypanosomiasis, HIV infection, and autoimmune disease (9, 14–21). Three single-nucleotide polymorphisms (SNPs) in promoters and a 5' untranslated region [(–550:rs11003125: G>C, H/L), (–221:rs7096206 G>C, Y/X), and (+4:rs7095891 C>T, P/Q)] are functionally characterized to alter the transcriptional levels and hence the serum MBL levels (22–24). In particular, promoter alleles –550L, –221X, and +4P are associated with lower MBL serum levels (13), whereas three nonsynonymous structural variants in exon1 of *MBL2* at codons 52, 54, and 57 of the collagen-like domain [(rs5030737: p.Arg52Cys:MBL2\*D), (rs1800450: p.Gly54Asp:MBL2\*B), and (rs1800451: p.Gly57Glu: MBL2\*C)] independently interfere with the formation of higher MBP oligomers, leading to alterations in the functional activity of the protein and their circulating levels (11, 25–30). Such structural variant alleles have been designated as O haplotype, whereas the normal *MBL2* allele has been designated as A haplotype (31). Further, these six SNPs (L/H, X/Y, P/Q, MBL2\*D, MBL2\*B, and MBL2\*C) have been shown to be in linkage disequilibrium (LD) and form eight common “secretor haplotypes” (HYPA, LYPA,

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LYQA, LXPA, HYPD, LYPB, LYQC, and LYPD) (8, 25, 32). These secretor haplotypes not only regulate the serum levels of functional MBL molecule but also modulate the complement activation (22, 26, 27, 33, 34). MBL2\*HYPA, LYQA, and LYPA are associated with high expression of MBL protein, whereas MBL2\*LXPA, HYPD, LYPB, and LYQC are associated with low expression of MBL protein (32, 35) and modulate the MBL concentration in healthy individuals from undetectable levels to 1,000-fold (36, 37).

To the best of our knowledge, no studies to date have investigated the association of MBL2 functional variants with malarial susceptibility in Indian populations. We sought here to investigate the contribution of polymorphisms in the entire MBL2 gene with *P. falciparum* malaria infection in well-defined malaria cases and in matched controls. Moreover, Indian populations are unique in their origin and have developed unique variations due to isolation from rest of the world for thousands of years (38, 39). Since the entire Indian subcontinent represents an area where malaria is endemic, we extended our study to 32 socially, linguistically, and geographically diverse endogamous Indian populations who live in this region.

## MATERIALS AND METHODS

**Sampling.** A total of 1,264 individuals were included in the present study, including 434 ethnically matched case-control samples that included asymptomatic individuals ( $n = 157$ , mean age of  $29.7 \pm 19.62$  years, and a male/female ratio of 2:65), individuals with mild malaria ( $n = 101$ , mean age of  $30.24 \pm 15.71$  years, and a male/female ratio of 67:34), and patients with severe malaria ( $n = 176$ , mean age of  $26.13 \pm 12.32$  years, and a male/female ratio of 103:73), as well as 830 individuals from 32 socially, linguistically, and geographically diverse endogamous populations of India. Patient samples were collected from those who were visiting or admitted with malaria for treatment at Ispat General Hospital, Rourkela, India, and Pt. Jawaharlal Nehru Memorial Medical College, Raipur, India. These are regions of India where *P. falciparum* is endemic. Microscopic results for all of the samples were reevaluated for the presence of *P. falciparum* by PCR using species-specific primers; targeting conserved the 18S rRNA gene of the parasite (40). Samples were clinically characterized according to World Health Organization (WHO) 2010 guidelines (41) and as described previously (42, 43). WHO guidelines define severe malaria by the presence of one or more of the facultative signs of severe malaria: cerebral malaria, convulsions, hypoglycemia, hemoglobinuria, prostration, and respiratory distress (acidotic breathing). Laboratory findings included hypoglycemia (blood glucose level of  $<2.2$  mmol/liter or  $<40$  mg/dl), metabolic acidosis (plasma bicarbonate level,  $<15$  mmol/liter), severe normocytic anemia (hemoglobin,  $<50$  g/liter; packed cell volume,  $<15\%$ ), hyperparasitemia ( $>2\%$  of infected red blood cells [RBCs] or 100,000 infected cells/ $\mu$ l in low-transmission areas or  $>5\%$  of infected RBCs or 250,000 infected cells/ $\mu$ l in high-transmission areas), hyperlactatemia (lactate,  $>5$  mmol/liter), renal impairment (serum creatinine,  $>265$   $\mu$ mol/liter), and a Blantyre coma score of  $\leq 3$ . Similarly, mild malaria is defined by a parasitemia of 1,000 to 50,000/ $\mu$ l on admission, no schizontemia, circulating leukocytes containing malarial pigment at  $<50/\mu$ l, nonhomozygous for hemoglobin S, hemoglobin level at  $>80$  g/liter, platelets at  $>50$ /nl, leukocytes at  $<12$ /nl, lactate at  $<3$  mmol/liter, and blood glucose at  $>50$  mg/dl. The asymptomatic control samples were collected from the same region where malaria is endemic and had no clinical signs of malaria irrespective of a few circulating parasites during sample collection. The clinical details of the patients included in the present study are given in Table 1.

**Ethics statement.** The institutional ethical committees for the Centre for Cellular and Molecular Biology, Hyderabad, India, the Ispat General Hospital, Rourkela, Orissa, India, and the Pt. Jawaharlal Nehru Memorial

TABLE 1 Clinical characterization of patient samples<sup>a</sup>

Clinical parameter <sup>b</sup>	Severe malaria ( $n = 176$ )	Mild malaria ( $n = 101$ )
Parasitemia (% of infected RBCs)	$2.53 \pm 1.16$	$1.08 \pm 0.69$
Hemoglobin (g/dl)	$5.68 \pm 1.37$	$8.93 \pm 2.40$
Blood glucose (mg/dl)	$43.92 \pm 13.81$	$80.74 \pm 19.36$
Blantyre coma score	$<3$	
No. of patients		
CM	71	
ARF	93	
IVH	83	
CM+ARF	44	
CM+IVH	51	
ARF+IVH	56	
CM+ARF+IVH	35	
Death	6	

<sup>a</sup> All patients with severe malaria were hospitalized due to complications related to one or more of the conditions listed in Table 1. Artesunate (2.4 mg/kg of body weight) was the main antimalarial treatment given intravenously, along with other supportive treatments, such as antibiotics, fluids, and ventilator support. Hemodialysis was performed, when required, in cases of renal failure. All mild malaria cases recovered with treatment uneventfully. All samples were checked for the HbS phenotype.

<sup>b</sup> CM, cerebral malaria; ARF, acute renal failure; IVH, intravascular hemolysis.

Medical College, Raipur, Chhattisgarh, India, all approved the study. Informed written consent was obtained from each individual.

**Gene sequencing.** Genomic DNA was isolated from whole blood according to a previously described protocol (44). The reference genomic sequences were obtained from the Ensembl database under Ensembl ID ENSG00000165471 ([www.ensembl.org](http://www.ensembl.org)). PCR and sequencing primers were designed using NCBI Primer-BLAST (<http://www.ncbi.nlm.nih.gov/tools/primer-blast>), MacVector (MacVector, Inc., USA), and Amplify 3X software (<http://engels.genetics.wisc.edu/amplify>). We resequenced 8.7 kb of the MBL2 gene, which includes  $\sim 1$  kb each of the upstream and downstream regions. The target regions of MBL2 were PCR amplified using the primer pairs MBL2\_F (5'-ATTACCCTGACCCAGGCCACTG TCTTCT-3') and MBL2\_R (5'-TACATAACATTGACCCCAAACCCATA GCAAG-3'). PCR amplifications were performed on a GeneAmp 9700 (Applied Biosystems) and using a thermal cycler (Eppendorf) with a long-range PCR kit (Qiagen, Germany) according to the manufacturer's protocol. The thermal cycling parameters consisted of an initial denaturation at 93°C for 3 min, followed by 35 cycles of 45 s at 93°C, 25 s at 66°C, and 9 min at 68°C.

Only the upstream promoter and first exonic regions ( $\sim 1.4$  kb) were resequenced for population samples. We PCR amplified the target region using two primer pairs—MBL2\_UPE1\_1F (5'-TCTGTGACTTTCCTCC ATTATCTGT-3')/MBL2\_UPE1\_1R (5'-TGGGAAGACTATAAACATGCTTTCCGG-3') and MBL2\_UPE1\_2F (5'-CATTCTCATTCCCTAAGCTA ACAGG-3')/MBL2\_UPE1\_2R (5'-CTGGCAAGACAACCTATTAGTCAC AA-3')—and an Emerald PCR master mix (TaKaRa). The thermal cycling parameters consisted of an initial denaturation at 95°C for 5 min, followed by 35 cycles of 30 s at 95°C, 25 s at 62°C, and 3 min at 72°C. PCR amplification was followed by Exo-SAP treatment (USB Corp., USA) according to the manufacturer's protocol. Exo-SAP-treated amplicons were sequenced directly by using a BigDye Terminator v3.1 cycle sequencing kit (Applied Biosystems) on an ABI 3730xl DNA analyzer. The 8.7-kb fragments were sequenced using 17 internal primers (see File S1 in the supplemental material). Sequence variations were identified by assembling DNA sequences with the reference sequence using AutoAssembler software (Applied Biosystems). Variations obtained were validated and reconfirmed in a subset of samples by resequencing and visual confirmation of electropherograms. Novel haplotypes were reconfirmed by subsequent resequencing.

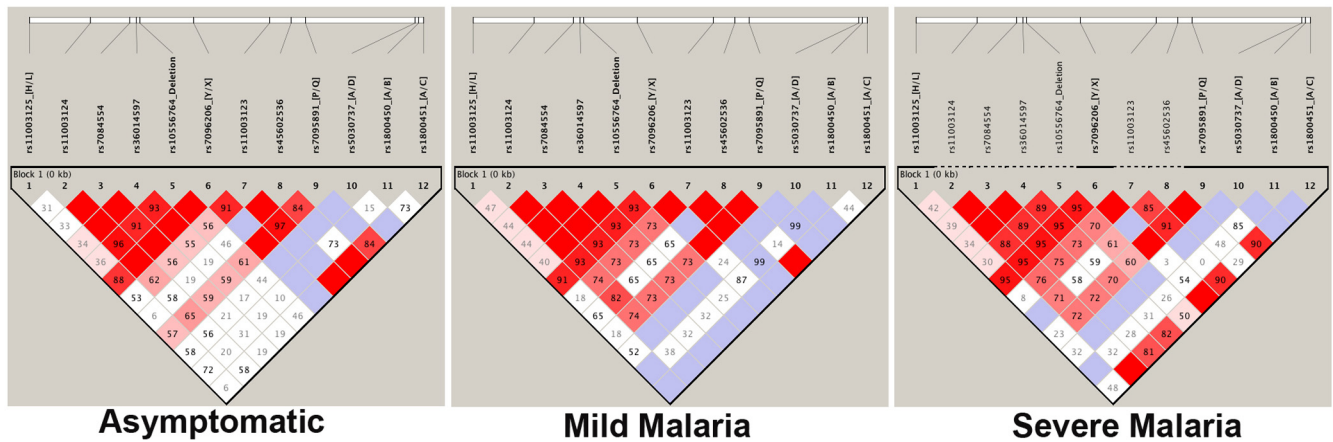


FIG 1 LD pattern of *MBL2* variants in asymptomatic, mild, and severe malaria groups. Empty squares indicate a high degree of LD ( $D' = 1$ ). Numbers indicate the  $D'$  value expressed as a percentile.

**Statistical analysis.** Risk estimates of severe and mild malaria were derived by comparison to the asymptomatic control group. We compared genotype, allele, and haplotype distributions for (i) asymptomatic versus mild-malaria groups, (ii) asymptomatic versus severe-malaria groups, and (iii) mild-malaria versus severe-malaria groups. The data were analyzed using Intercooled Stata v9.1, and the level of significance was set to a  $P$  value of  $<0.05$ . The allele and genotype frequencies were analyzed by simple allele/genotype counting and an expectation-maximum algorithm. The significance of deviations from Hardy-Weinberg (HW) equilibrium was tested using plink v1.7 (45). The allele and genotype distributions and tests of association were performed using plink. A two-tailed Fisher exact test was performed to determine the differences in allele frequencies and genotype distributions. Linkage disequilibrium (LD) analysis was performed by Haploview v4.2 (46). Bonferroni corrections were applied for multiple comparisons.

## RESULTS

Sequencing of the complete *MBL2* gene, along with  $\sim 1$  kb each of the upstream and downstream, led to the detection of a total of 30 polymorphic loci: 29 SNPs and a 6-bp deletion in the promoter region. We observed a novel SNP in the 3' untranslated region at the genomic coordinate position g.54526021C>A. The genotype and allele distribution of all the variations among the case-control groups is summarized in File S2 in the supplemental material. All observed and investigated loci were in HW equilibrium. The detected variants after exon1 do not significantly contribute in any comparisons. The LD between the promoter and exon1 *MBL2* variants in different groups is shown in Fig. 1. The promoter region of the asymptomatic group was in stronger LD compared to the cases of mild and severe malaria. The genotype and allele distributions observed in different groups and their respective associations are summarized in Table 2.

**Odds of malaria (asymptomatic versus mild malaria).** The genotype frequencies of the promoter polymorphism  $-221G>C$  (rs7096206; *MBL2*:Y/X) differ significantly between the asymptomatic and mild-malaria groups (Table 2). The frequency of heterozygous genotype YX ( $-221GC$ ) was significantly higher in subjects with mild malaria ( $P = 0.0021$ ) compared to asymptomatic controls (after multiple correction) (Table 2 and Fig. 2). Similarly, the frequency of allele X was significantly higher in patients with mild malaria compared to asymptomatic controls (asymptomatic versus mild,  $P = 0.0036$ ; Table 2 and Fig. 2). These findings sug-

gests that the  $-221GC$  heterozygous bears an increased risk toward mild malaria. None of the other variations were associated with mild malaria (Table 2 and see File S2 in the supplemental material).

Earlier studies demonstrated that the *MBL2* serum levels are modulated by  $-221G/C$  variant and three structural variants of exon1. Therefore, we studied the genotypes of variants in promoter ( $-221G/C$ ) and exon1 (codons 52 [*MBL2*\*D], 54 [*MBL2*\*B], and 57 [*MBL2*\*C]) together and investigated for their influence in serum MBL levels (high [YA/YA], intermediate [YA/YO, XA/XA, and XA/YA], and low [YO/YO, XA/YO, and XA/XO] MBL producers). We observed that the diplotype YA/YO was significantly low in frequency in the group with mild malaria ( $P = 0.033$ ) compared to asymptomatic individuals (Table 3 and Fig. 2).

The reconstructed *MBL2* secretor haplotype distributions and their comparison among case-control groups are summarized in Table 4. Apart from the eight well-known *MBL2* secretor haplotypes, we observed four novel *MBL2* secretor haplotypes: *MBL2*\*HYQA, *MBL2*\*HYQC, *MBL2*\*LXPB, and *MBL2*\*LYQB. We observed that the low-serum *MBL2* haplotype *MBL2*\*LXPA was significantly more frequent in the group with mild malaria compared to the asymptomatic group ( $P = 0.0045$ ) (Table 4). None of the other reconstructed haplotypes contributed significantly to association.

**Odds of severe malaria (asymptomatic versus severe malaria).** Although the frequency of the heterozygous genotype  $-221G>C$  (Y/X) was significantly higher in subjects with mild malaria ( $P = 0.0021$ ), we did not determine its contribution to severe malaria compared to the asymptomatic control (after multiple correction) (Table 2). However, the frequency of allele  $-221X$  was significantly higher in patients with severe malaria compared to asymptomatic controls ( $P = 0.02$ ) (Table 2).

The distribution of the structural variant *MBL2*\*B (rs1800451: codon 54, G>A) differed significantly between asymptomatic and severe malaria (Table 2). The heterozygous genotype GA (*MBL2*\*AB) was observed significantly at high levels ( $P = 0.03$ ) compared to asymptomatic controls (Table 2). Further, the frequency of minor allele A (*MBL2*\*B) was significantly greater in cases of severe malaria ( $P = 0.036$ ) (after multiple corrections) (Table 2). These findings suggest that the structural variant *MBL2*\*B, which interferes with oligomerization and hence the low

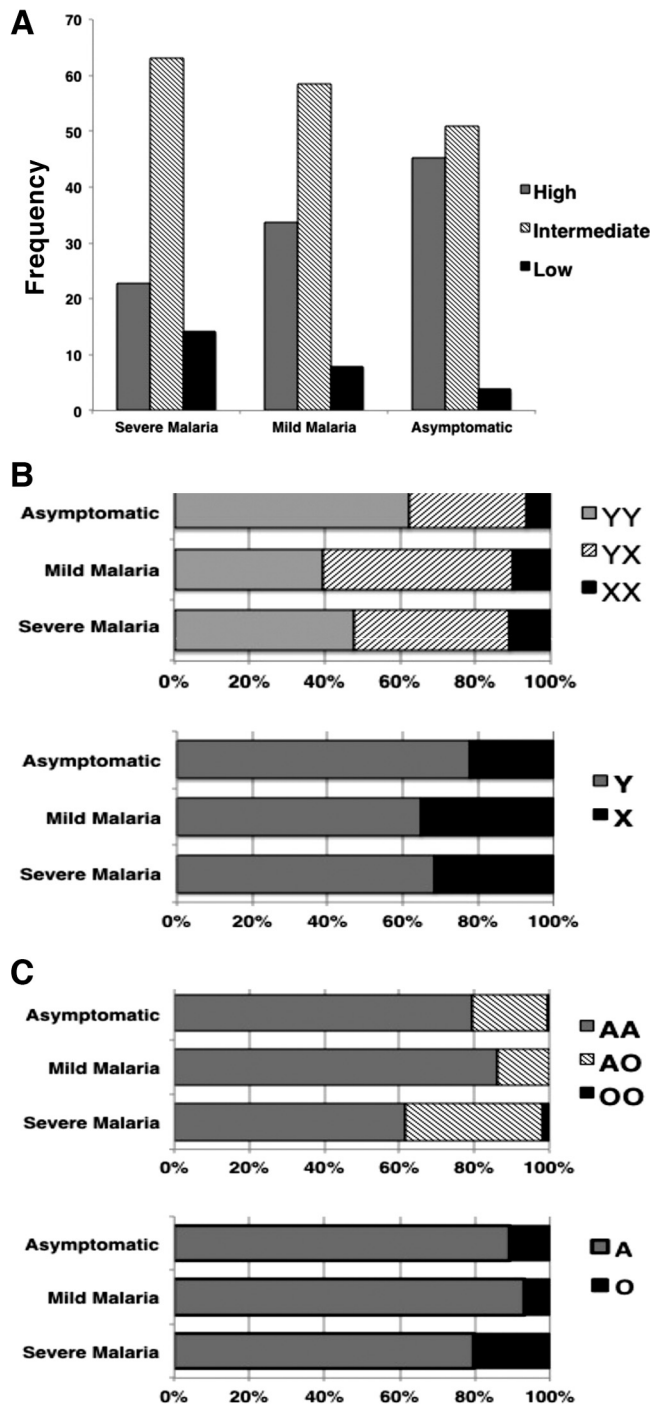
TABLE 2 *MBL2* polymorphisms present in the promoter and first exonic region and comparison of genotypes and alleles among case-control groups

SNP position	No. (%) of patients			Genotype comparison <sup>a</sup>					
	Asymptomatic (n = 157)	Mild malaria (n = 101)	Severe malaria (n = 176)	Asymptomatic vs mild		Asymptomatic vs severe		Mild vs severe	
				OR (95% CI)	P	OR (95% CI)	P	OR (95% CI)	P
<b>rs11003125 (-550C/G)</b>									
CC (LL)	93 (59.2)	66 (65.4)	104 (59.1)		R		R		R
GC (HL)	51 (32.5)	29 (28.7)	59 (33.5)		NS		NS		NS
GG (HH)	13 (8.3)	6 (5.9)	13 (7.4)		NS		NS		NS
C (L)	237 (75.5)	161 (79.7)	267 (75.9)		R		R		R
G (H)	77 (24.5)	41 (20.3)	85 (24.1)		NS		NS		NS
<b>rs7096006 (-221G/C)</b>									
GG (YY)	98 (62.4)	40 (39.6)	84 (47.7)		R		R		R
GC (YX)	49 (31.2)	51 (50.5)	73 (41.5)	2.55 (1.4–4.5)	0.0021		NS		NS
CC (XX)	10 (6.4)	10 (9.9)	19 (10.8)		NS		NS		NS
G (Y)	245 (78)	131 (64.9)	241 (68.5)		R		R		R
C (X)	69 (22)	71 (35.1)	111 (31.5)	1.9 (1.3–2.9)	0.0036	1.6 (1.1–2.4)	0.02		NS
<b>rs7095891 (+4C/T)</b>									
CC (PP)	68 (43.3)	56 (55.4)	90 (51.1)		R		R		R
CT (PQ)	69 (44)	35 (34.6)	77 (43.8)		NS		NS		NS
TT (QQ)	20 (12.7)	10 (9.9)	9 (5.1)		NS	0.34 (0.13–0.85)	0.043		NS
C (P)	205 (65.3)	147 (72.8)	257 (73)		R		R		R
T (Q)	109 (34.7)	55 (27.2)	95 (27)		NS		NS		NS
<b>MBL2* exon1 (codon 52)</b>									
C>T									
CC	153 (97.5)	98 (97)	162 (92.1)		R		R		R
CT	4 (2.6)	3 (3)	14 (8)		NS		NS		NS
TT	0 (0)	0 (0)	0 (0)		NS		NS		NS
C	310 (98.7)	199 (98.5)	338 (96)		R		R		R
T	4 (1.3)	3 (1.5)	14 (4)		NS		NS		NS
<b>MBL2* exon1 (codon 54)</b>									
G>A									
GG	140 (89.2)	91 (90.1)	138 (78.4)		R		R		R
GA	16 (10.2)	10 (9.9)	36 (20.5)		NS	2.3 (1.2–4.6)	0.03		NS
AA	1 (0.6)	0 (0)	2 (1.1)		NS		NS		NS
G	296 (94.3)	192 (95)	312 (88.6)		R		R		R
A	18 (5.7)	10 (5)	40 (11.4)		NS	2.1 (1.1–3.99)	0.036	2.5 (1.2–5.6)	0.039
<b>MBL2* exon1 (codon 57)</b>									
G>A									
GG	144 (91.7)	99 (98)	159 (90.3)		R		R		R
GA	13 (8.3)	2 (2)	16 (9.1)		NS		NS		NS
AA	0 (0)	0 (0)	1 (0.6)		NS		NS		NS
G	301 (95.9)	200 (99)	334 (94.9)		R		R		R
A	13 (4.1)	2 (1)	18 (5.1)		NS		NS	5.4 (1.3–48)	0.045
<b>Mbl2* exon1 codons</b>									
52+54+57									
AA	125 (79.6)	87 (86.1)	108 (61.4)		R		R		R
AO	31 (19.7)	14 (13.9)	65 (36.9)		NS	2.4 (1.4–4.1)	0.0012	3.7 (1.9–7.7)	0.00008
OO	1 (0.6)	0 (0)	3 (1.7)		NS		NS		NS
A	281 (89.5)	188 (93.1)	281 (79.8)		R		R		R
O	33 (10.5)	14 (6.9)	71 (20.2)		NS	2.1 (1.4–3.5)	0.0018	3.4 (1.8–6.7)	0.000045

<sup>a</sup> NS, not significant; R, reference. P values were corrected for multiple comparisons by Bonferroni's method.

level of functional MBL, is a risk factor for *P. falciparum* malaria (Table 2). Further, when we grouped all three structural variants of exon1 (*MBL2*\*D/B/C), we observed a significantly higher frequency of heterozygous genotype *MBL2*\*AO ( $P = 0.0021$ ) com-

pared to the control group. We also observed similar trends in allelic distribution. The frequency of allele *MBL2*\*O was significantly higher in the group with severe malaria ( $P = 0.0018$ ) compared to the asymptomatic controls (Table 2).



**FIG 2** Distribution of *MBL2* variants in various case-control groups. (A to C) Distribution of high-, intermediate-, and low-serum *MBL2* haplotypes (A), XY variants (B), and exon1 structural variants A and O allele (C) [(-221: rs7096206 G>C, Y/X), (rs5030737: p.Arg52Cys:*MBL2*\*D), (rs1800450: p.Gly54Asp:*MBL2*\*B), and (rs1800451: p.Gly57Glu:*MBL2*\*C)]. Exon1 structural variant alleles (D, B, and C) have been designated as the O haplotype, whereas the normal *MBL2* allele is designated as A.

Diplotype analysis shows that the frequency of the high-MBL-producer diplotype (YA/YA) was significantly low ( $P = 0.00003$ ) in the severe-malaria group compared to asymptomatic controls (Table 3 and Fig. 2). Similarly, the high *MBL2* secretor haplotypes

*MBL2*\*LYPA and *MBL2*\*LYQA were significantly less frequent in the severe-malaria group compared to the asymptomatic group (*MBL2*\*LYPA [ $P = 0.015$ ] and *MBL2*\*LYQA [ $P = 0.048$ ]) (Table 4). Further, we grouped the *MBL2* haplotypes into two groups: the secretor haplotypes that lead to a high level of functional MBL (*HYPA* + *LYQA* + *LYPA* + *HYQA*) made up one group, and those that lead to a low level (*LXPA* + *LYPB* + *LYQC* + *HYPD* + *LYPD* + *HYQC* + *LXPB* + *LYQB*) made up another. We observed a significant difference between asymptomatic subjects and those with severe malaria. The high-MBL-producing haplotypes were significantly less frequent ( $P = 0.00002$ ), while the low-serum-MBL haplotypes were significantly more frequent ( $P = 0.00002$ ) in the group with severe malaria compared to the asymptomatic controls (Table 4).

**Odds of malaria progression (mild malaria versus severe malaria).** The exon1 structural variants (*MBL2*\*D, *MBL2*\*B, and *MBL2*\*C) differ significantly between mild and severe malaria cases. We observed that the frequency of the minor allele A at codon 54 (*MBL2*\*B) was higher in severe malaria ( $P = 0.039$ ) compared to mild malaria (Table 2). Similarly, the frequency of minor allele A at codon 57 (*MBL2*\*C) was significantly higher in severe-malaria cases than in cases of mild malaria ( $P = 0.045$ ) (Table 2). The combined genotypes of three structural variants of exon1 show that the allele O (*MBL2*\*D, *MBL2*\*B, and *MBL2*\*C) was significantly more frequent in individuals with severe malaria ( $P = 0.000045$ ) compared to those with mild malaria (Table 2).

In line to this observation, the frequency of the high-serum-MBL secretor haplotype *MBL2*\*LYPA was lower in cases of severe malaria than in the mild-malaria group ( $P = 0.02$ ) (Table 4). This suggests that the exon1 structural variant that affects the oligomerization increases the risk of severe malaria.

***MBL2* genotype and haplotype distribution in ethnic populations of India.** To characterize *MBL2* polymorphisms in different ethnic populations of India, we extended our study to 830 individuals from 32 socially, linguistically, and geographically diverse endogamous populations of India. The population details and genotype distribution of functional variants (-550C>G, -221G>C, +4C>T, *MBL2*\*D, *MBL2*\*B, and *MBL2*\*C) that affects serum MBL levels are summarized Table 5. The frequency of low *MBL2* secretor haplotypes varies significantly between Indian populations (low, 0 to 62.5%) (Fig. 3). We did not observe low-*MBL2* haplotypes in two of the investigated tribal populations (Subba and Gond), whereas an ancestral tribal population Juang revealed a high (62.5%) frequency. However, the distribution of the AO and XY diplotypes showed that the high prevalence of the low-*MBL2* haplotype in Juang is mainly due to promoter variant (X, 62.50%) and not because of structural variants (O, 17.19%) (Fig. 3). Similarly, Subba tribes had high frequencies of A (78.13%) and Y (68.75%) alleles, and the resulting major genotype YA (Fig. 3). Further, the *MBL2* exon1 variant *MBL2*\*B is the prevalent form of structural variant in most of the Indian populations (Fig. 3).

**DISCUSSION**

Mannose-binding lectin (MBL) is a soluble innate immune pathogen recognition protein that plays a vital role in defense during the early phase of infection (9). Several studies have shown that *MBL2* genetic variants regulate the MBL serum levels in different autoimmune diseases and infectious diseases, such as malaria, leishmaniasis, leprosy, tuberculosis, schistosomiasis,

**TABLE 3** Distribution of combined genotypes for variants at *-221G/C* and exon1 at codons 52 (*MBL2\*D*), 54 (*MBL2\*B*), and 57 (*MBL2\*C*)

Promoter (-221) + exon1	No. (%) of patients			Genotype comparison <sup>a</sup>					
				Asymptomatic vs mild		Asymptomatic vs severe		Mild vs severe	
	Asymptomatic (n = 157)	Mild malaria (n = 101)	Severe malaria (n = 176)	OR (95% CI)	P	OR (95% CI)	P	OR (95% CI)	P
YA/YA	71 (45.22)	34 (33.66)	40 (22.73)		NS	0.35 (0.21–0.58)	0.00003		NS
YA/YO	26 (16.56)	6 (5.94)	43 (24.43)	0.3 (0.1–0.8)	0.033		NS	5.1 (2.04–15.2)	0.0003
YO/YO	1 (0.64)	0	1 (0.57)		NA		NS		NA
XA/YA	44 (28.02)	43 (42.57)	50 (28.41)		NS		NS	0.53 (0.31–0.92)	0.05
XA/XA	10 (6.37)	10 (9.90)	18 (10.23)		NS		NS		NS
XA/YO	5 (3.18)	8 (7.92)	21 (11.93)		NS	4.1 (1.4–14.3)	0.01		NS
XA/XO	0	0	1 (0.57)		NA		NA		NA
XO/YO	0	0	2 (1.13)		NA		NA		NA

<sup>a</sup> NS, not significant; NA, not applicable. P values were corrected for multiple comparisons by Bonferroni's method.

trypanosomiasis, and HIV infection (9, 14–21). The clinical significance of *MBL2* variants varies with the disease context in different world populations (21, 47–49).

We observed significant differences in genotype and allele frequencies among asymptomatic subjects and in patients with mild and severe malaria. The genotype and allele distribution of promoter polymorphism *-221XY* differed significantly between the asymptomatic and mild-malaria groups, as well as between the asymptomatic and severe-malaria groups, whereas the exon1 structural variants (*MBL2\*B*) were associated with severe malaria only. Individuals homozygous for structural variants in exon1 (*MBL2\*B*, *MBL2\*C*, and *MBL2\*D*) were rare. None of the other variations were associated with either mild or severe malaria. Earlier studies have documented the role of other functional *MBL2*

promoter polymorphism (*-550HL*) in several diseases (21, 50); however, we did not find any statistical significance in our study. We observed that the *-221C(X)* allele was found at significantly higher frequencies in malaria cases, which increases the risk for clinical malaria. We also observed that the structural *MBL2* variants were significantly less frequent in asymptomatic subjects but higher in the severe-malaria group, showing that individuals with structural *MBL2* variants are at higher risk for severe malaria. Thus, our findings suggest that *MBL2* variants that decrease the plasma level of the functional MBL protein or encode dysfunctional proteins make their carriers susceptible to malaria. This result is in accordance with previous studies, which demonstrated that the low-serum-MBL genotype is a risk factor for malarial infection (5, 9, 32, 51); however, a study performed in Gambia

**TABLE 4** Distribution and comparison of *MBL2* secretor haplotypes in case and control groups

Haplotype or comparison <sup>a</sup>	No. (%) of patients			Genotype comparison <sup>b</sup>					
				Asymptomatic vs mild		Asymptomatic vs severe		Mild vs severe	
	Asymptomatic (n = 314)	Mild malaria (n = 202)	Severe malaria (n = 352)	OR (95% CI)	P	OR (95% CI)	P	OR (95% CI)	P
<b>High MBL secretor haplotypes</b>									
<i>MBL2*HYPA</i>	66 (21.02)	34 (16.83)	70 (19.88)		NS		NS		NS
<i>MBL2*LYPA</i>	48 (15.28)	31 (15.34)	29 (8.23)		NS	0.5 (0.3–0.8)	0.015	0.5 (0.27–0.85)	0.02
<i>MBL2*LYQA</i>	94 (29.93)	51 (25.24)	76 (21.59)		NS	0.6 (0.45–0.9)	0.048		NS
<i>MBL2*HYQA</i> †	2 (0.66)	3 (1.48)	0		NS		NA		NA
<b>Low MBL secretor haplotypes</b>									
<i>MBL2*LXPA</i>	68 (21.65)	70 (34.65)	106 (30.11)	1.9 (0.3–2.9)	0.0045	1.56 (1.1–2.2)	0.042		NS
<i>MBL2*LYPB</i>	18 (5.73)	9 (4.45)	32 (9.09)		NS		NS		NS
<i>MBL2*LYQC</i> †	8 (2.55)	0	12 (3.41)		NA		NS		NA
<i>MBL2*HYPD</i>	4 (1.27)	2 (0.99)	8 (2.27)		NS		NS		NS
<i>MBL2*HYQC</i>	6 (1.91)	2 (0.99)	5 (1.42)		NS		NS		NS
<i>MBL2*LYPD</i>	0	0	6 (1.70)		NA		NA		NA
<i>MBL2*LXPB</i> †	0	0	4 (1.13)		NA		NA		NA
<i>MBL2*LYQB</i> †	0	0	4 (1.13)		NA		NA		NA
<b>High vs low</b>									
High‡	210 (66.88)	119 (58.91)	175 (49.71)		0.075	0.5 (0.45–0.68)	0.00002		NS
Low§	104 (33.12)	83 (41.09)	177 (50.28)		0.075	2 (1.5–2.8)	0.00002		NS

<sup>a</sup> †, Novel secretor haplotypes observed in this study; ‡, high expression of *MBL2\** (*HYPA* + *LYQA* + *LYPA* + *HYQA*); §, low expression of *MBL2\** (*LXPA* + *LYPB* + *LYQC* + *HYPD* + *LYPD* + *HYQC* + *LXPB* + *LYQB*).

<sup>b</sup> NS, not significant; NA, not applicable. P values were corrected for multiple comparisons by Bonferroni's method.

TABLE 5 Geographical origin, ethnicity, linguistic affiliation, and genotype distributions of populations in the present study

Serial no.	Population	Social designation	Language family	State	Indian region <sup>a</sup>	Sample size	Genotype distribution <sup>b</sup>					
							-550C>G CC/CG/GG	-221G>C GG/CG/CC	+4C>T CC/CT/TT	MBL2*D CC/CT/TT	MBL2*B GG/AG/AA	MBL2*C GG/AG/AA
1	Thapa	Caste	Tibeto-Burman	Nepal	Nepal	32	12:18:02	16:16:00	15:14:03	31:01:00	24:08:00	30:02:00
2	Bharia	Tribe	Dravidian	Madhya Pradesh	CI	31	18:12:01	6:20:05	20:11:00	31:00:00	22:09:00	30:01:00
3	Kol	Tribe	Indo-European	Madhya Pradesh	CI	29	15:12:02	7:12:10	22:07:00	27:02:00	24:05:00	29:00:00
4	Kurmi	Caste	Indo-European	Madhya Pradesh	CI	26	10:15:01	16:09:01	15:08:03	25:01:00	19:07:00	24:02:00
5	Kanyakubja	Caste	Indo-European	Madhya Pradesh	CI	28	19:08:01	13:14:01	14:06:08	28:00:00	24:04:00	28:00:00
6	Juang	ATP <sup>c</sup>	Austro-Asiatic	Orissa	CI	32	23:09:00	2:20:10	12:18:02	31:01:00	23:09:00	29:03:00
7	Maria	Tribe	Austro-Asiatic	Orissa	CI	80	41:36:03	42:31:07	32:38:10	76:04:00	61:18:01	71:09:00
8	Bho	Tribe	Indo-European	Uttaranchal	NI	24	15:07:02	13:10:01	7:15:02	24:00:00	20:04:00	20:04:00
9	Oraon	Tribe	Indo-European	Jharkhand	CI	28	12:13:03	15:10:03	14:11:03	27:01:00	21:06:01	26:02:00
10	Munda	Tribe	Austro-Asiatic	Jharkhand	CI	30	15:13:02	12:15:03	15:13:02	30:00:00	24:06:00	28:02:00
11	Santhal	Tribe	Austro-Asiatic	Jharkhand	CI	41	23:15:03	17:17:07	20:14:07	41:00:00	33:08:00	37:04:00
12	Rajgond	Tribe	Indo-European	Madhya Pradesh	CI	26	5:14:07	17:09:00	13:12:01	20:06:00	19:07:00	22:04:00
13	Sonker	Caste	Indo-European	Madhya Pradesh	CI	27	11:14:02	18:07:02	9:14:04	25:02:00	19:08:00	26:01:00
14	Thoti	Caste	Dravidian	Andhra Pradesh	AP	27	12:13:02	17:07:03	15:09:03	27:00:00	21:05:01	24:03:00
15	Banjara	Nomadic	Indo-European	Rajasthan	WI	35	18:13:04	24:09:02	17:17:01	34:01:00	25:10:00	34:01:00
16	Bhil	Tribe	Indo-European	Gujarat	WI	21	12:07:02	12:06:03	11:10:00	21:00:00	16:04:01	21:00:00
17	Changapa	Tribe	Tibeto-Burman	North East	NE	20	7:06:07	11:05:04	10:10:00	16:04:00	16:04:00	20:00:00
18	ChakhesNaga	ATP	Tibeto-Burman	Nagaland	NE	18	11:06:01	14:03:01	12:06:00	18:00:00	12:06:00	18:00:00
19	Mizo	ATP	Tibeto-Burman	Mizoram	NE	20	7:12:01	13:06:01	9:10:01	20:00:00	13:07:00	19:01:00
20	Aonaga	ATP	Tibeto-Burman	Nagaland	NE	19	3:12:04	12:06:01	17:02:00	19:00:00	15:04:00	19:00:00
21	Nagaseema	Tribe	Tibeto-Burman	Nagaland	NE	24	7:12:05	12:10:02	10:11:03	24:00:00	18:06:00	23:01:00
22	Nyshi	Tribe	Tibeto-Burman	Arunachal Pradesh	NE	17	0:10:07	12:03:02	11:04:02	15:02:00	13:04:00	15:02:00
23	Bhootia	Tribe	Tibeto-Burman	Sikkim	NE	20	2:06:12	16:04:00	15:05:00	16:04:00	15:05:00	20:00:00
24	Khasi	Tribe	Tibeto-Burman	Meghalaya	NE	22	3:13:06	15:07:00	13:08:01	21:01:00	15:07:00	22:00:00
25	Puma	Tribe	Tibeto-Burman	Sikkim	NE	15	7:04:04	10:05:00	15:00:00	15:00:00	6:07:02	15:00:00
26	Sherpa	Tribe	Tibeto-Burman	Sikkim	NE	21	6:12:03	16:05:00	8:12:01	21:00:00	16:05:00	14:06:01
27	Subba	Tribe	Tibeto-Burman	Darjeeling/WB	NE	16	0:12:04	10:06:00	12:04:00	15:01:00	14:02:00	12:04:00
28	Birhor	ATP	Austro-Asiatic	Jharkhand	CI	31	13:10:08	21:07:03	16:10:05	31:00:00	25:05:01	18:10:03
29	Lambadi	Tribe	Dravidian	Andhra Pradesh	AP	19	8:09:02	13:04:02	11:06:02	9:00:00	11:07:01	11:06:02
30	Kabutarayan	Caste	Indo-European	Uttar Pradesh	NI	28	15:10:03	15:11:02	15:12:01	28:00:00	24:04:00	26:02:00
31	Gond	Tribe	Dravidian	Chhattisgarh	CI	10	3:04:03	6:03:01	5:04:01	10:00:00	10:00:00	10:00:00
32	G.Andamanese	ATP	Andamanese	Andaman	Andaman	13	1:02:10	6:05:02	13:00:00	11:02:00	13:00:00	13:00:00
Total						830						

<sup>a</sup> AP, Andhra Pradesh; CI, central India; NI, north India; NE, northeast India; WI, west India; WB, west Bengal.

<sup>b</sup> Genotype distributions were in HW equilibrium at 0.05 level.

<sup>c</sup> ATP, ancestral primitive tribe.

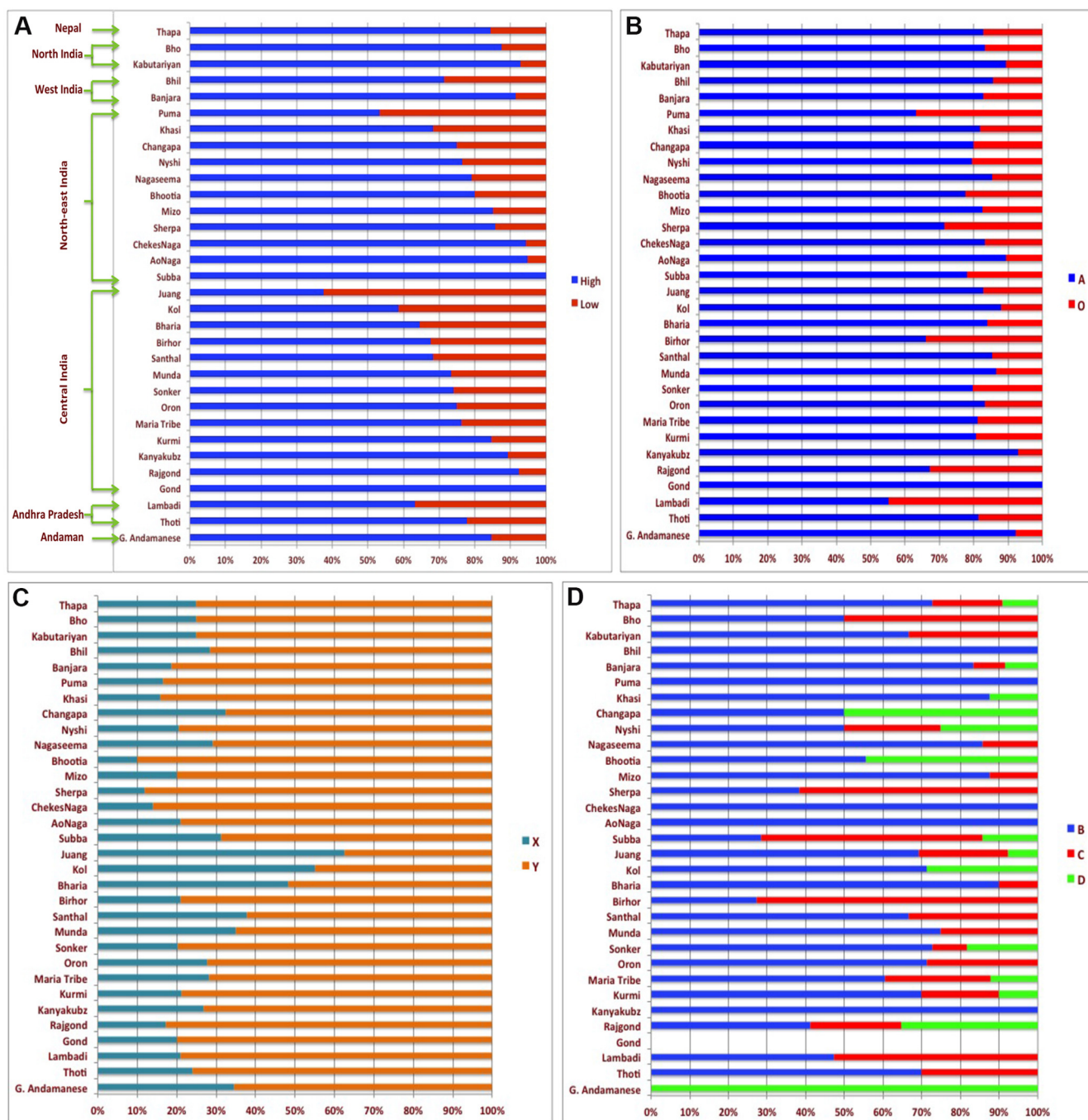
(47) failed to replicate this association. The serum level of functional MBL is mainly regulated by the promoter polymorphism -221XY and exon1 structural variant A/O (O: *MBL2*\*D, *MBL2*\*B, and *MBL2*\*C). We found all three structural variants in our study. The genotype YA/YA was found to be significantly less common in the severe-malaria group than in asymptomatic controls, whereas genotype XA/YO was more common. We also observed that the genotype YA/YO was significantly less common in mild-malaria cases compared to asymptomatic subjects. These findings indicate that a low-serum-MBL diplotype might be a risk factor for severe malaria while the intermediate-serum-MBL diplotype might be a risk factor for mild malaria.

Several studies have shown the effects of *MBL2* secretor haplotypes in disease association studies (9, 21, 32). Our investigation of the *MBL2* secretor haplotype also showed significant differences in haplotype carriage between various case-control groups. Stratification of our cohort based on *MBL2* haplotypes showed that the haplotypes *MBL2*\*LYPA and *MBL2*\*LYQA were significantly less common in the severe-malaria group, whereas haplotype *MBL2*\*LXPA was significantly less common in the mild-malaria group. This again elucidates that the individuals with the high-serum-MBL haplotype have low risk for malaria. In addition to the eight common haplotypes reported previously (9), we observed here four novel *MBL2* secretor haplotypes, *MBL2*\*HYQA, *MBL2*\*HYQC, *MBL2*\*LXPB, and *MBL2*\*LYQB, although at much lower frequencies that were independently reconfirmed by subsequent sequencing procedures. The exceptional uncharacterized

secretor haplotypes *MBL2*\*LYQB and *MBL2*\*HXPA have also been reported in Bakola Pygmy and Moroccan Berber populations, respectively (52).

Apart from structural variants, we did not observe any other variations that were statistically significant between the severe and mild-malaria groups. The comparison shows that the structural variants are over-represented in severe cases, while the normal variants are over-represented in the mild-malaria group, suggesting that the severity of *P. falciparum* malaria is inversely proportional to the serum level of functional MBL.

In the present study, among 32 Indian populations across India, we observed that the frequency of *MBL2* haplotypes, which leads to low level of serum MBL, varies drastically from 0% in Subba and Gond to 62.5% in an ancestral tribal population in Juang. The prevalence of low-MBL haplotypes in Juang is mainly due to the prevalence of X and is not due to structural variants. Further, high- and low-serum-MBL haplotypes are not restricted to a particular geographic region. In the same geographic region, populations show varied prevalences of *MBL2* haplotypes and, hence, may show different levels of susceptibility to malaria and other infectious diseases. These findings are in agreement with the genetic structure of Indian populations, who maintain their unique genetic architecture, mainly due to endogamy marriage practice over the last several thousand years. This has been well supported by our earlier studies with mtDNA, Y chromosome, and autosomal genetic markers (38, 39, 42, 44). We also observed all three structural variants in exon1 (*MBL2*\*D, *MBL2*\*B, and



**FIG 3** Distribution of *MBL2* variants in 32 Indian populations across India. (A to D) Distribution of *MBL2* haplotypes (A), AO variants (B), XY variants (C), and exon1 structural variants *MBL2*\*B, *MBL2*\*C and *MBL2*\*D (D) [(-221:rs7096206 G>C, Y/X), (rs5030737: p.Arg52Cys:*MBL2*\*D), (rs1800450: p.Gly54Asp: *MBL2*\*B) and (rs1800451: p.Gly57Glu:*MBL2*\*C)]. Structural variant alleles (D, B, and C) have been designated as O haplotype, whereas the normal *MBL2* allele is designated as A.

*MBL2*\*C) in Indian populations. In addition, the *MBL2*\*D/B/C (>20%) and *MBL2*\*Y (>75%) variants were observed at higher frequencies in Lambadi, Rajgond, Birhor, Subba, Sherpa, Bhootia, and Puma populations, suggesting a possible selective heterozygous advantage. *MBL2*\*B is the most prevalent structural variant in Indian populations, in contrast to *MBL2*\*C in African populations.

We investigated whether *MBL2* functional variants can mod-

ulate or alter serum MBL levels; hence, individuals harboring these variants, secretor, or low or high producers have either increased or decreased risk *per se*. In conclusion, functional *MBL2* genetic variants are significantly associated with malaria pathogenesis in Indian populations. To the best of our knowledge, this is the first study of its kind, in which we studied the *MBL2* variations in detail in diverse Indian populations. An individual with mild



malaria can progress into a more severe form and so this is true for severe to mild malaria or asymptomatic to mild malaria. Therefore, further studies on ethnically different populations, as well as larger case-control and cohort studies, are strongly recommended in order to obtain better estimates of malarial risks associated with the *MBL2* polymorphism.

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A.N.J. and K.T. planned and designed the experiment. S.S.P. and P.K.P. collected patient blood samples and carried out clinical assessments. A.N.J. analyzed the results. A.N.J., P.S., and V.K.S. prepared samples and performed experiments. T.P.V. rechecked the statistical analysis. A.N.J. prepared the manuscript. A.N.J., P.G.K., L.S., and K.T. prepared the final manuscript. L.S. and K.T. provided all of the materials and facilities and supervised the entire study.

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