Genetic Polymorphisms of Mannose-Binding Lectin Do Not Influence Placental Malaria but Are Associated with Preterm Deliveries[∇]

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During pregnancy, *Plasmodium falciparum*-infected erythrocytes (IE) sequester in the placenta where they induce pathology and increase the risk of low-birth-weight (LBW) babies. The innate immune mediator, mannose-binding lectin (MBL), enhances phagocytosis of pathogens. Since MBL is reported to bind to IE, we hypothesized that it might aid in clearance of IE from the placenta, thereby reducing the risk of LBW babies. To test this hypothesis, molecular genotyping was used to detect polymorphisms at codon 57 (A/C) in exon 1 of *MBL2* in 401 pregnant Cameroonian women, with or without placental malaria, who had LBW and normal-weight babies. Polymorphisms in the promoter region at positions –550 (H/L), –221 (X/Y), and +4 (P/Q) were also determined, and plasma MBL levels were measured during pregnancy and at delivery. The expected correlation between genotype and plasma MBL levels was confirmed. However, asymptomatic infections were not associated with an increase in MBL levels in the peripheral blood, and MBL levels were similar in the placental and cord blood of women with or without placental malaria at delivery. There was no evidence that MBL levels at delivery were associated with malaria-related poor pregnancy outcomes. Women with the LXPA haplotype, however, were more likely to have LBW babies, but the risk was not related to malaria. These results do not support the hypothesis that MBL aids in the clearance of parasites from the placenta but suggest that Cameroonian women with LXPA are at risk of having LBW babies due to other causes.

Women who become infected with *Plasmodium falciparum* during pregnancy are at an increased risk of anemia and poor pregnancy outcomes (5, 25). One reason for these complications is that *P. falciparum*-infected erythrocytes (IE) sequester in the intervillous space (IVS) of the placenta (9). As a result, monocytes and macrophages are attracted to the IVS (30, 36), where they stimulate an inflammatory response, produce placental pathology (42), and increase the risk of low-birth-weight (LBW) babies due to intrauterine growth reduction and premature deliveries (reviewed in reference 6). Thus, elimination of IE from the IVS is of great importance.

Little is known about the role of innate immune cells and factors in eliminating IE from the IVS. An acute-phase protein that might be important is mannose-binding lectin (MBL), since it promotes opsonophagocytosis and activates the MBL pathway upon binding to oligosaccharides on pathogens (12, 17). MBL is reported to bind to the surface of IE and possibly merozoites, although the ligand involved remains undefined (10, 15). In addition, *MBL2* genetic deficiencies in children are associated with severe malaria (4, 11). Therefore, it is possible that MBL aids in eliminating IE by enhancing phagocytosis, leading to reduced pathology and risk of LBW babies.

Plasma levels of MBL are influenced by three functional single nucleotide polymorphisms (SNPs) in exon 1 of the *MBL2* gene at codons 52, 54, and 57 (D, B, and C, respectively) (18, 20, 21, 35). These SNPs lead to a disruption in the arrangement of MBL oligomers, producing a nonfunctional lectin that is rapidly degraded after disassembly (37). As a result, serum MBL levels are lower in individuals who are heterozygous or homozygous for the variant alleles than in those with the wild type (21, 37). The frequencies of these SNPs vary between ethnic populations. For example, in Africa variant C predominates (~26%), whereas it is relatively uncommon (<1%) in Europe (21, 35). In addition, several SNPs in the promoter region at positions –550 (H/L) and –221 (X/Y) and the 5'-untranslated portion at position +4 (P/Q) also influence the amount of MBL produced (21). For instance, individuals carrying the haplotype LX in the promoter region have lower MBL expression than individuals carrying the haplotypes HY or LY (21). Adults living in sub-Saharan Africa are likely to have a wide range of plasma MBL levels due both to genotype diversity and to frequent microbial infections. Accordingly, in the present study both molecular genotyping of SNPs and serum levels of MBL were assessed.

The purpose of the present study was to evaluate the importance of MBL in placental malaria. Specifically, we sought to determine whether (i) MBL levels are increased in the peripheral blood in response to *P. falciparum* infection; (ii) high MBL levels in the IVS were associated with the absence of, or low, placental parasitemias; and (iii) pregnant women with genotypes associated with low MBL production were at an increased risk of delivering LBW babies when they had placental malaria.

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TABLE 1. Women ($n = 401$) selected for *MBL2* (codon 57) genotyping^a

Parameter	NBW		LBW	
	PM−	PM+	PM−	PM+
No. of subjects selected	173	108	69	51
Mean age (yr) $\pm$ SD	24.6 $\pm$ 5.7	24.4 $\pm$ 5.4	23.1 $\pm$ 5.6	23.0 $\pm$ 5.1
% Subjects with primigravidae	47.4	37.0	47.8	54.0
% Subjects with placental parasitemia	0	5.0 $\pm$ 10.6	0	6.5 $\pm$ 12.8
Mean gestational age (wk) $\pm$ SD at delivery	40.1 $\pm$ 1.9	39.4 $\pm$ 5.6	35.0 $\pm$ 4.1	35.9 $\pm$ 4.7
Mean birth wt (g) $\pm$ SD	3,378 $\pm$ 426	3,233 $\pm$ 436	1,918 $\pm$ 421	1,960 $\pm$ 419

^a The composition of the group of 152 women selected according to exon 1 genotype for the *MBL2* promoter and 5' untranslated region genotyping was as follows (n = the number of subjects matching each description). A/A: NBW PM−, $n = 21$; NBW PM+, $n = 22$; LBW PM−, $n = 19$; LBW PM+, $n = 17$. A/C + C/C: NBW PM−, $n = 22$; NBW PM+, $n = 18$; LBW PM−, $n = 20$; LBW PM+, $n = 13$.

MATERIALS AND METHODS

Selection of samples. Maternal buffy coat cells and plasma were selected from a panel of ~2,000 samples obtained from consecutive deliveries in Yaoundé, Cameroon (38) (Table 1). Initially, 401 women were selected, including all women who had LBW babies ($n = 120$) for whom buffy coat cells were available. Among these women, 50 had placental malaria. An additional 281 samples were selected from women with normal-birth-weight (NBW) babies (i.e., >2,500 g) based on frequency matching. That is, for each woman in the LBW group, the next two women with NBW deliveries with approximately the same age and malarial status were selected. Characteristics of the 401 selected women are shown in Table 1. Gestational age was based on (i) self-reported last menstrual period, (ii) sonogram data when available, and (iii) assessment at delivery by the attending physician. All 401 women were genotyped for the A/C polymorphism in exon 1 codon 57 of *MBL2*. Once their genotype was known, 152 women were further selected for genotyping of SNPs in the promoter region of *MBL2* (Table 1, footnote a) and corresponding MBL levels were determined. First, an approximately equal number of women were selected based on their Exon 1 genotypes (A/A [$n = 79$] and A/C and C/C [$n = 73$]). Then, women within each genotype were selected based on pregnancy outcome and malaria status as shown in Table 1, footnote a. The 152 women in the subgroup were similar to those in the larger group ($n = 401$) with respect to age, placental parasitemia, anemia, and gestational age but had a higher proportion of primigravidae with LBW babies. In an additional study, the amount of MBL in paired maternal peripheral blood, placental IVS blood, and fetal cord blood was compared using samples from 49 of the 401 women ($n = 35$ LBW babies; $n = 14$ NBW babies).

Peripheral blood samples were also collected from 54 women during the course of pregnancy. Among the women, 34 lived in Yaoundé where transmission of *P. falciparum* is low, i.e., ~13 infectious bites/person/year (24), and 20 women lived in the rural village of Ngali II, where the transmission rate is ~265 infectious bites/person/year. Buffy coat cells were used to determine polymorphisms in exon 1 at codon 57. Samples of peripheral blood collected from each woman during the first, second, and third trimesters and at delivery (i.e., four samples per woman) were analyzed for MBL levels. The results are based on 193 available samples. Asymptomatic women who were blood smear positive for *P. falciparum* were prescribed antimalarial treatment according to the Ministry of Health's policy, which included chloroquine, quinine (Quinimax; Sanofi-Aventis, France), amodiaquine, or artesunate (Plasmodim; Mepha Ltd., Switzerland).

Ethical clearance and informed consent. This study was approved by the Institutional Review Board, Georgetown University and the National Ethical Committee, Ministry of Public Health, Cameroon. Written or oral informed consent was obtained from each woman.

Determination of parasitemia. Thick and thin blood smears of samples collected during pregnancy and at delivery were stained with Diff-Quik (Baxter Scientific Products, Miami, FL) and examined for the presence of *P. falciparum*. Cord blood and biopsies of the placental tissue were also collected. First, the umbilical cord was clamped and blood was collected by using a needle and syringe. Once the cord blood was been removed, a section of tissue (2 by 2 by 2 cm) located midway between the center and outer edge of the placental disc was biopsied from the maternal side. A portion of the tissue was used to prepare impression smears that were stained with Diff-Quik and examined for parasites. The remainder of the tissue was fixed in buffered formalin, histological sections were prepared, and stained with hematoxylin-eosin and Giemsa. Placental malaria was defined as detection of parasites in either impression smears or histological sections. In addition to detecting parasites, histosections were examined for pathology related to chronic (e.g., parasites plus macrophages and extensive

hemozoin pigment) or past infections (no parasites but residual hemozoin pigment), i.e., the Bulmer score (7). This analysis was not possible on all histological sections due to poor fixation, including formalin crystals that are indistinguishable from hemozoin. Maternal IVS placental blood was collected from the biopsy site. As previously reported, the placental blood samples were minimally contaminated with cord blood since $6.8\% \pm 3.4\%$ fetal-hemoglobin-containing erythrocytes were detected in the blood samples (36). Parasitemia was recorded as percentage of infected erythrocytes. Blood samples from the 54 women monitored throughout the course of pregnancy were also tested by PCR for the presence of *P. falciparum* as described by Snounou et al. (34). Women who were blood smear negative but PCR positive were classified as having submicroscopic infections.

Genotyping of *MBL2*. DNA was purified from 455 women (i.e., 401 women enrolled at delivery, and 54 women were monitored throughout pregnancy). Maternal DNA from ~55 μ l of buffy coat cells was extracted by using a flexible solid-phase system incorporated in capture columns (Generation capture column kit; Gentra Systems, Minneapolis, MN).

The *MBL2* gene was genotyped by using a combination of PCR-based methods. The polymorphism at codon 57 of exon 1 (A/C) (dbSNP ID rs1800451) was determined by PCR-restriction fragment length polymorphism (RFLP) using the method of Lipscombe et al. (18) and modified by Mombo et al. (27). The *cis/trans* location of SNPs localized in the gene promoter at positions −550 (L/H) (dbSNP ID rs11003125) and −221 (X/Y) (dbSNP ID rs7096206) was determined by PCR-sequence-specific primer (21). In addition, the PCR-sequence-specific primer method optimized by Steffensen et al. (35) was used to genotype L, H, X, and Y independently in order to confirm SNPs for each woman. Finally, the P and Q alleles at position +4 (dbSNP ID rs7095891) were detected by RFLP performed on site-directed mutagenesis-PCR (21).

All PCRs were performed using a total volume of 20 to 50 μ l containing 100 to 150 ng of genomic DNA, 1.5 to 1.7 mM MgCl₂ (Applied Biosystems, Foster City, CA), 0.25 to 0.5 μ M concentrations of specific primers (Invitrogen), 0.2 mM concentrations of each deoxynucleotide triphosphate (Invitrogen), 1 U of AmpliTaq DNA polymerase (Applied Biosystems), and 1 $\times$ of PCR buffer provided in the PCR kit. Immediately after DNA amplification, the RFLP methods used to detect the alleles C, P, and Q employed endonucleases MboII, SacI, and HindIII, respectively (New England Biolabs, Beverly, MA) (18, 20). PCR products were separated on a 2% agarose gel (UltraPure; Invitrogen), stained with 1 μ g of ethidium bromide (Bio-Rad, Hercules, CA)/ml, and visualized with UV light.

Quantification of serum levels of MBL. MBL concentrations in 613 plasma samples from 206 pregnant women and 49 fetal cord blood samples were measured by using a semiquantitative ligand-lectin solid-phase enzyme-linked immunoassay from Sanquin Reagents (Amsterdam, The Netherlands). The assay only detects multimeric MBL. Plates were coated overnight with mannan in 0.05 M carbonate-bicarbonate buffer (pH 9.6). Plasma samples were diluted 1:20 with MBL-binding buffer. After incubation, anti-human MBL antibodies coupled to horseradish peroxidase were added, and binding was detected using 3,3',5,5'-tetramethylbenzidine (Sigma-Aldrich), followed by the addition of 100 μ l of Stop Reagent (Sigma-Aldrich). Absorbance was read at 450 nm (model Elx808; BioTek, Winooski, VT), and a standard curve was plotted from standards provided in the kit.

Statistical analysis. Pearson correlation coefficient was used to compare MBL levels among peripheral, IVS, and cord blood, and the Spearman correlation coefficient was used to assess correlations of malaria parasitemia and MBL level in the IVS. The between-group differences in the percentage of placental malaria

TABLE 2. Frequency of the alleles at codon 57 in exon 1 of *MBL2*

Genotype or allele	No. of women (<i>n</i> = 455)	Frequency (%)	Median MBL concn (ng/ml) ^a
Genotype			
A/A	304	66.8	1,171
A/C	144	31.7	101
C/C	7	1.5	0
Allele			
A		82.5	
C		17.5	

^a Median values are based on *n* = 111 for A/A women, *n* = 82 for A/C women, and *n* = 7 for C/C women. MBL levels differed significantly among the groups ($P < 0.001$).

infection, LBW, and other categorical variables were compared by using the chi-square test. The between-group differences in MBL levels at delivery were tested by using the Wilcoxon rank-sum or Kruskal-Wallis test as appropriate. The between-group comparisons in repeated measures of MBL level during pregnancy were made by using a likelihood ratio test (LRT) based on a mixed model with an unstructured covariance structure among repeated measures. All of the statistical analyses were conducted by using SAS software (v9.1; SAS, Cary, NC).

RESULTS

Frequency of polymorphisms at codon 57 of exon 1 in *MBL2* and corresponding MBL levels. Among the 455 selected women (*n* = 401 selected plus 54 women monitored throughout pregnancy), 66.8% were homozygous wild type (A/A), 31.7% were A/C, and 1.5% were C/C (Table 2). The overall allelic frequency of the C variant was 17.5%, which is in agreement with that reported in the Cameroonian population (26). The frequency was marginally outside of Hardy-Weinberg equilibrium ($P = 0.09$, chi-square test). This was not unexpected, since only women with successful pregnancies were included in the study. Women with SNPs in exon 1 of the *MBL2* gene are known to produce lower amounts of MBL (18) and to be more susceptible to early termination of pregnancy due to recurrent miscarriages (8, 13, 16). Since only 7 women were homozygous C/C, they were combined with women who were A/C in further analyses.

To confirm that the C polymorphism influenced functional MBL levels, the concentration of MBL was determined using 152 peripheral blood samples (see Table 1, footnote *a*) collected at delivery (Table 2). Although the level of serum MBL varied greatly among the women, a 10-fold difference in median MBL levels was found among women with the wild-type A/A genotype (median = 1,171 ng/ml) and women with the A/C (101 ng/ml) and C/C genotypes (0 ng/ml) ($P < 0.001$; Wilcoxon rank-sum test). Thus, the polymorphism at codon 57 was associated with different levels of plasma MBL.

Plasma levels of MBL during the course of pregnancy in Cameroonian women. MBL levels were measured in peripheral samples collected during the first, second, and third trimesters and at delivery from 34 women in Yaoundé and 20 women in Ngali II (Fig. 1). At both sites, women with the A/A genotype had higher levels of MBL at all four time points (median values > 900 ng/ml) (Fig. 1A) compared to A/C+C/C women (median values < 600 ng/ml) (Fig. 1B) ($P = 0.018$, LRT). A/A women living in the village had higher median

MBL levels than women living in the city, but the difference was not significant ($P = 0.329$, LRT). No significant change in MBL levels was found in the peripheral blood during the first, second, and third trimesters and at delivery term ($P = 0.59$, adjusted for site and genotype) (Fig. 1). Therefore, MBL levels remained relatively constant throughout pregnancy, which is consistent with results reported for European women (14, 40).

Asymptomatic *P. falciparum* infections did not significantly alter peripheral MBL levels. The ability of *P. falciparum* infections to stimulate MBL production during the course of pregnancy was assessed in 54 women (Fig. 2 and 3). First, peripheral MBL levels for the same woman were compared when she was malaria positive versus when she was negative (Fig. 2). The lowest amount of MBL detected when the woman was PCR negative for malaria served as the baseline. Changes in peripheral blood MBL for women who were PCR negative throughout pregnancy (Fig. 2A) and those who became slide positive or had submicroscopic infections are shown in Fig. 2B.

FIG. 1. MBL levels in plasma during the course of pregnancy. Peripheral plasma was collected every other month from women in Yaoundé (*n* = 34) and in Ngali II (*n* = 20). Each data point is based on A/A Yaoundé (*n* = 15 to 21 samples), A/C Yaoundé (*n* = 10 to 12 samples), A/A Ngali II (*n* = 8 to 15 samples), or Ngali II A/C+C/C (*n* = 3 to 5 samples). Box-whisker plots depict the 10th, 25th, 50th, 75th, and 90th percentiles. MBL levels were higher in A/A than in A/C+C/C subjects ($P = 0.018$).

FIG. 2. Changes in MBL levels in the peripheral blood during the course of pregnancy. (A) Modulation of MBL levels in women ($n = 6$) who were PCR negative for *P. falciparum* at all time points. (B) MBL levels in women ($n = 6$) when they were (■) slide positive, (▲) had submicroscopic infection, or (●) were slide and PCR negative for malaria.

A one- to threefold modulation in MBL levels was observed in women who remained PCR negative (Fig. 2A). No consistent change in MBL levels was seen when women became slide positive or had submicroscopic infections compared to when they were PCR negative (Fig. 2B). Second, since plasma MBL levels remained stable throughout pregnancy (Fig. 1), the levels of MBL were compared among samples collected when women were blood smear positive, had submicroscopic infections, and were negative by PCR at various points during pregnancy (Fig. 3). High MBL levels were detected in some slide-positive samples from A/A women, but the difference was not significant compared to PCR-negative genotype-matched samples from these women ($P = 0.215$). Thus, no significant increase in MBL was detected during asymptomatic *P. falciparum* infections.

MBL levels in the peripheral blood at delivery. At delivery, MBL levels in the peripheral blood were similar between women who had placental malaria ($n = 70$) and those who did not ($n = 82$) (537.9 ng/ml versus 489.1 ng/ml, respectively, $P = 0.686$ [Wilcoxon rank-sum test]). Likewise, peripheral blood

MBL levels in primigravidae were similar in women with or without placental malaria (median 433.4 versus 613.6, respectively, $P = 0.585$), in primi- and multigravidae mothers with placental malaria (median MBL 433.4 versus 578.3, $P = 0.372$), and in women with or without placental malaria who had LBW babies (13.5% versus 15.5%). Importantly, no association between MBL levels in the peripheral blood at delivery and placental parasitemias was found (Spearman correlation coefficient $r = 0.087$, $P = 0.287$). Moreover, there was no difference in MBL levels in mothers who had LBW ($n = 69$) and NBW ($n = 83$) babies (371.1 ng/ml versus 550.4 ng/ml, $P = 0.199$).

Plasma MBL levels in the peripheral, placental, and cord blood at delivery. Plasma MBL levels were measured in 49 women (peripheral and placental IVS) and in the cord blood of their babies at delivery ($n = 35$ [LBW] and $n = 14$ [NBW]). Regardless of the malarial status of the woman, the MBL levels were consistently lowest in maternal peripheral blood, intermediate in the IVS, and highest in cord blood ($P < 0.01$, Wilcoxon signed rank test). A moderate correlation was found between levels of MBL in the peripheral blood and IVS (Pear-

FIG. 3. Influence of *P. falciparum* infection on plasma MBL levels. Levels of MBL in plasma collected when women were malaria-positive and negative ($n = 193$ samples). The results for A/A and A/C+C/C are based on 24 and 11 slide-positive samples, 35 and 13 samples from submicroscopic infections, and 70 and 40 PCR-negative samples, respectively. Box-whisker plots depict the 10th, 25th, 50th, 75th, and 90th percentiles. No differences in MBL levels were found.

son correlation coefficient = 0.595) and between amounts in IVS and cord blood (Pearson correlation coefficient = 0.697), but only a weak correlation was found between peripheral and cord blood (Pearson correlation coefficient = 0.296). Thus, a

difference in circulating MBL levels was found between the three compartments. Based on data from these 49 mothers and off-springs, no difference in MBL levels was found in IVS placental ($P =$

FIG. 4. MBL levels in maternal peripheral blood, IVS blood, and fetal cord blood. Box-whisker plots depict the 10th, 25th, 50th, 75th, and 90th percentiles. A significant difference in MBL levels was found between maternal peripheral blood, blood in the IVS, and fetal cord blood ($P < 0.01$, Wilcoxon signed rank test). However, MBL levels were similar in these compartments for women who were infected with *P. falciparum* and those who were not (DNS). $n = 49$ women and cord samples.

TABLE 3. Frequency of MBL genotypes among the 152 selected women and corresponding plasma MBL levels

Subject group	No. of women	Median MBL (ng/ml) (Q1-Q3) ^a
Selected women with the A/A genotype (<i>n</i> = 79)		
LYQA/LYPA	6	1,974 (973–3,779)
LYQA/LYQA	19	1,856 (937–3,256)
LYQA/HYPA	17	1,526 (572–3,137)
LXPA/HYPA	3	1,422 (1,250–5,309)
LYQA/LXPA	16	952 (436–4,197)
LXPA/LXPA	3	795 (456–2,926)
LYPA/HYPA	3	329 (92–433)
LYPA/LYPA	9	199 (67–709)
HYPA/HYPA	2	
LYPA/LXPA	1	
Selected women with A/C or C/C genotype (<i>n</i> = 73)		
LYQC/LYQA	34	206 (0–623)
LYQC/HYPA	16	38 (0–1,058)
LYQC/LXPA	18	14 (0–233)
LYQC/LYPA	3	0 (0–0)
LYQC/LYQC	2	

^a Q1 and Q3 represent the medians of the lower and upper quartiles, respectively.

0.691, Wilcoxon rank-sum test) or cord blood ($P = 0.838$, Wilcoxon rank-sum test) between babies born to mothers with or without placental malaria (Fig. 4), and there was no correlation between MBL levels in the IVS and placental parasitemias (Spearman correlation coefficient $r = 0.05$). Based on results from 49 women, placental malaria does not appear to influence MBL levels in either IVS or fetal cord blood at delivery.

No association between SNPs in codon 57 of exon 1 and placental malaria or LBW babies. In order to test the influence of MBL SNPs on delivery outcomes, 120 women with LBW and 281 NBW babies were selected (Table 1). The results show that a similar percentage of A/A and A/C+C/C mothers had placental malaria (39% versus 41%, respectively) and LBW babies (A/A = 31.0% versus A/C+C/C = 27.3%). Information on Bulmer scores was available for 111 of the 401 women. The proportions of women with A/A and A/C+A/C genotypes was similar among those who had chronic ($n = 25$ women; 50% A/A versus 50% A/C+C/C) or past placental infections ($n = 32$ women; 46.9% versus 53.1%). Thus, SNPs in codon 57 of exon 1 that resulted in low or high levels of functional MBL (Table 2) did not influence the prevalence of placental malarial or LBW babies.

Polymorphisms in the promoter region. The presence of polymorphisms in *MBL2* at positions –550 (H/L), –221 (X/Y), and +4 (P/Q) were determined using DNA from 152 women selected based on their genotype at codon 57 (A/A, A/C, or C/C), delivery outcome, and placental malaria (Table 1, footnote *a*). The results showed that the most frequent haplotype in the cohort was LYQA (36.5% [111 of 304 possible haplotypes]), which is a haplotype that has been reported to be common in Africa (21). This was followed by LYQC (24.7%), LXPA (14.5%), HYPA (14.1%), and LYPA (10.2%) (Table 3).

Among A/A women, those with the genotypes LYQA/

LYPA, LYQA/LYQA, LYQA/HYPA, and LXPA/HYPA had high levels of serum MBL (range of medians of 1,422 to 1,974 ng/ml), those with LYQA/LXPA had intermediate levels of MBL (median, 952 ng/ml), and those with LYPA/LYPA had low levels of MBL (median, 199 ng/ml) (Table 3). The influence of these SNPs was less apparent in women with the A/C+C/C genotypes, due to their lower levels of MBL. Thus, SNPs located outside the coding region directly influenced serum MBL levels.

Association of maternal haplotypes and MBL levels with LBW babies. Mothers with the LXPA haplotype, in association with either LYQA or LYQC, were at a higher risk of having LBW babies than women with the other haplotypes combined ($P = 0.001$, chi-square test) (Table 4). The median gestation period for women with the LXPA haplotype was 37.6 weeks compared to 40 weeks for the mothers who do not have the LXPA haplotype ($P = 0.007$, Wilcoxon rank-sum test). Babies born before the completion of the 37th week of gestation are classified as premature; therefore, most of the LBW babies were due to premature deliveries. LBW babies born to mothers with LXPA had a lower birth weight (1,900 and 1,800 g) compared to babies of mothers with the other haplotypes ($P < 0.001$ for all women; $P = 0.005$ in placental malaria-negative women, and $P = 0.002$ in malaria-positive women [Wilcoxon rank-sum test]). Although a higher proportion of the selected mothers expressing LXPA/LYQA (75%) and LXPA/LYQC (50%) had placental malaria compared to the other mothers shown in Table 4 (<48%), the difference was not statistically significant ($P = 0.388$, chi-square test). On the other hand, mothers with the HYPA/A genotype, considered in multiple studies to be associated with high levels of MBL (21), had the lowest percentage of LBW babies (12%) compared to women with the other major genotypes combined (42%) ($P = 0.005$, chi-square test) (Table 4).

Although an association between maternal genotypes and LBW babies was found, no association was observed with MBL levels. That is, no difference was found in MBL levels in either maternal peripheral blood, IVS placental blood, or cord blood between LBW and NBW deliveries ($P = 0.352$, $P = 0.064$, and $P = 0.217$, respectively). Furthermore, no difference in MBL levels in maternal peripheral, IVS, or cord blood was found among NBW and LBW infants due to premature deliveries or intrauterine growth reduction (peripheral blood [$P = 0.138$], IVS [$P = 0.162$], and cord blood [$P = 0.434$], respectively). In the present study, SNPs in the promoter region, but not MBL levels, were associated with the frequency of LBW babies.

DISCUSSION

Since MBL binds to the surface of IE, we hypothesized that MBL might aid in the clearance of parasites sequestered in the placenta, especially early in first pregnancies before mothers produce antibodies that block cytoadherence. Our results agree with previous reports on the prevalence of the exon 1 codon 57 SNP of *MBL2* in Cameroonians and other African populations (22, 26). The results also verify the influence of SNPs on MBL levels (Tables 2 and 3) (21, 22). However, the data do not support a beneficial role for MBL in placental malaria. First, during pregnancy plasma MBL levels were not enhanced in women with asymptomatic *P. falciparum* infec-

TABLE 4. Influence of the six most common maternal *MBL2* genotypes on the prevalence of LBW babies

Genotype	No. of women ^a	% LBW babies ^b	Median wk of gestation (Q1-Q3) ^c	Median birth wt (g) (Q1-Q3)		PER	Median MBL (ng/ml) (Q1-Q3) ^e		% of with PLA MAL
				LBW babies ^d	NBW babies		PLA	CORD	
LYQAL/YQA	19	42.1 (8/19)	39.8 (39.1-40.3)	1,900 (1,450-2,275)	3,300 (3,050-3,960)	1,856 (937-3,256)	1,309 (1,035-1,910)	1,450 (1,200-1,996)	47.4
HYPA/LYQA	17	11.8 (2/17)	39.8 (38.7-41.3)	1,312 (1,173-1,450)	3,380 (3,350-3,700)	1,526 (572-3,137)	961 (507-1,729)	274 (254-705)	47.1
LXPA/LYQA	16	75.0* (12/16)	37.5† (31.8-40.2)	1,900‡ (1,500-2,315)	2,650 (2,535-2,865)	952 (436-4,197)	1,491 (296-1,682)	1,972 (805-2,125)	75.0
LXPA/LYQC	18	61.1* (11/18)	37.7† (34.0-39.6)	1,800‡ (1,600-2,000)	3,110 (2,500-3,550)	14 (0-233)	217 (1,181-387)	458 (243-2,512)	50.0
HYPA/LYQC	16	43.8 (7/16)	39.5 (36.3-41.5)	2,200 (2,100-2,470)	3,430 (3,010-3,630)	38 (0-1,056)	160 (56-321)	283 (136-1,618)	37.5
LYQAL/YQC	34	35.3 (12/34)	40.0 (37.1-41.1)	2,100 (1,825-2,380)	3,350 (3,100-3,670)	206 (0-628)	826 (454-1,114)	1,398 (356-1,907)	36.4

^a Of 152 women, 120 had the six genotypes listed; the other 32 women had nine different genotypes.

^b Values are based on a total of 152 women who were genotyped. *, $P = 0.001$ (Wilcoxon rank-sum test). Values in parentheses indicate the number of LBW babies/the total number of subjects in the group.

^c †, $P = 0.007$ (Wilcoxon rank-sum test).

^d ‡, $P < 0.0001$ (Wilcoxon rank-sum test).

^e PER, peripheral blood; PLA, placental blood; CORD, cord blood.

tions (Fig. 2 and 3). Second, at delivery MBL levels were similar in the peripheral and placental blood of women with or without placental malaria ($P = 0.13$ and $P = 0.69$, respectively). Third, placental malaria in primi- and multigravidae was not correlated with either SNPs in codon 57, SNPs in the promoter region, or plasma MBL levels. Finally, no association with either SNPs or MBL levels and malaria-associated pregnancy outcomes was observed. Therefore, it seems unlikely that MBL is a major innate immune mediator in placental malaria.

Although MBL has been studied for over 10 years, a protective role for this lectin in malaria has been difficult to establish. In vitro MBL binds to undefined parasite cytoplasmic proteins and the surface of IE (10, 15). The nature of the ligand bound by this lectin remains unclear, since the plasmodial genome lacks most glycosyltransferases required for N glycosylation (33). Few studies have shown that *P. falciparum* antigens activate the MB-lectin pathway. The pathway is not triggered during the liver stage of malaria (32). However, elevated MBL levels have been found in sera of Gabonese children with severe, but not mild, malaria (4, 19), and a 1.5- to 2-fold increase in MBL was detected in the sera of Sudanese adults (39) with severe malaria. In the current study, pregnant women had asymptomatic and submicroscopic infections, and their MBL levels did not increase compared to when they were PCR negative for malaria (Fig. 2 and 3). Unlike in the Sudanese study, a 1.5- to 2.0-increase in MBL did not constitute a positive response in our study because one- to threefold changes in MBL levels were observed in pregnant women who remained malaria negative throughout pregnancy (Fig. 2A). To our knowledge, no study has found an association between MBL and decreased parasitemias or protection from malaria. Similarly, no association between placental parasitemias and MBL levels was found in pregnant Cameroonian women. Thus, additional information is needed about the role of MBL in protection.

SNPs in the *MBL2* gene have been linked to increased severity of disease in young children, especially in children who are homozygous for the polymorphism (10, 27). For example, a new variant in exon 1, called g.797C, which is in absolute linkage disequilibrium with the C variant, is overrepresented in Gabonese children with severe malaria (4). Furthermore, the haplotype LYQC is more prevalent in young Ghanaian children with severe malaria than in healthy aparasitemic children (11), but the association was not observed in children older than 2 years, suggesting that age-dependent adaptive immunity to malaria acquired by older children supersedes the beneficial effects of MBL. On the other hand, an extensive study by Bellamy et al. found no association between the prevalence of variants B and C and severe malaria in Gambian children (2); data specific for children <2 years were not evaluated. Taken together, data suggest that SNPs in *MBL2* may be associated with severe malaria in young children but not with asymptomatic infections in children (27) or adults, including pregnant women, who have substantial levels of acquired immunity.

The prevalence of LBW babies was higher in Cameroonian women who had the LXPA haplotype compared to women carrying the other haplotypes (Table 4). In accordance with previous studies (21), we found LXPA to be associated with lower MBL levels due to SNPs in the promoter region. This is

the first study to find an association between polymorphisms in the *MBL2* promoter region and LBW babies. The majority of babies whose mothers had the haplotype LXPA were born prior to 38 weeks of gestation (Table 4) and thus had LBW due to prematurity. Previously, genetic variants in exon 1 have been linked with spontaneous abortions and miscarriages (8, 13, 16) and shown to have an influence on preterm deliveries (1, 41). Annells et al. (1) found an increased risk of having deliveries at <29 weeks in women with the variant B, an allele associated with low MBL production. Interestingly, van de Geijn et al. (41) reported an association between high MBL levels and shorter gestational periods in normal deliveries. In our study, an association was found with genotype but not with MBL levels. Clearly, additional studies are needed to evaluate the effect of MBL genotype on pregnancy outcome independent of malaria.

In the present study, polymorphisms in the promoter region, but not exon 1, were associated with LBW babies. This result was unexpected since SNPs in both regions resulted in lower MBL levels (Tables 2 and 3). Several explanations are proposed. First, SNPs in the *MBL2* gene other than those evaluated here may be involved, e.g., variants in exon 4 could contribute to structural abnormalities (3, 4). Second, LXPA might be in linkage disequilibrium with other genetic polymorphisms within the *MBL2* gene or perhaps linked to other genes encoding other innate immune components. Finally, proinflammatory cytokines have a major influence on MBL production, especially in individuals with the genotype XA/YA (4). Therefore, future studies on the interaction of LXPA, infectious diseases and their effects on premature deliveries are warranted.

This is the first study to measure the concentration of MBL in the IVS. Although maternal peripheral blood continuously circulates through the IVS, MBL levels were often higher in the IVS than in the peripheral blood (Fig. 4). The increase in MBL is most likely of fetal origin since mRNA for the lectin is present in amniotic fluids (23), and MBL is expressed by first trimester cytotrophoblasts and mesenchymal cells (13). Recently, Oudshoorn et al. reported that MBL amounts were equal in umbilical venous and arterial blood and that the levels in cord blood differ from those in maternal peripheral blood (31). Likewise, in the present study, only a weak correlation was observed in MBL levels between peripheral and cord blood (Pearson correlation coefficient = 0.296). These data suggest that the fetus produces MBL either constitutively or in response to "foreign stimuli." In either case, release of MBL into the IVS could protect the fetus against pathogens that might otherwise cause local pathology. Interestingly, fetal trophoblasts also produce sFlt1, a molecule with anti-inflammatory and antiangiogenic activity that is coded for by the *FLT1* gene. Recent studies show that in women with placental malaria, the maternal *FLT1* genotype was associated with LBW deliveries in first time mothers (29) and that sFlt1 was produced by trophoblasts in response to placental malaria (28). However, the potential biological role of sFlt1 in the context of placental malaria remains unclear. In our study, enhanced MBL levels in cord blood were not associated with placental malaria, but it is possible the fetus was exposed to other pathogens or immunostimulatory molecules in utero that induced the MBL response. Clearly, additional studies are needed to

determine whether MBL can be triggered by pathogens that cross the placenta or by cytokines produced in the IVS that induce signaling across fetal villous membranes.

The initial hypothesis was that women with low MBL-producing genotypes would maintain placental infections for a longer period than women who produced high levels of MBL upon infection and thus be at a higher risk of poor pregnancy outcomes. We found no convincing evidence to support the hypothesis. These results do not rule out a role for MBL in malaria. All of the women in the study had very low peripheral parasitemias and asymptomatic infections, and placental histology revealed that very few women had active placental malaria at delivery, a condition more likely to be associated with elevated MBL levels. Longitudinal studies comparing when a woman is infected, the resulting placental histopathology, and amount of MBL in cord blood are needed to fully assess the role of MBL. Additional studies are also warranted to establish the biological basis for the association between LXPA and LBW babies.

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REFERENCES

- Annells, M. F., P. H. Hart, C. G. Mullighan, S. L. Heatley, J. S. Robinson, P. Bardy, and H. M. McDonald. 2004. Interleukins-1, -4, -6, -10, tumor necrosis factor transforming growth factor- β , FAS, and mannose-binding protein C gene polymorphisms in Australian women: risk of preterm birth. *Am. J. Obstet. Gynecol.* **191**:2056–2067.
- Bellamy, R., C. Ruwende, K. P. McAdam, M. Thursz, M. Sumiya, J. Summerfield, S. C. Gilbert, T. Corrah, D. Kwiatkowski, H. C. Whittle, and A. V. Hill. 1998. Mannose binding protein deficiency is not associated with malaria, hepatitis B carriage nor tuberculosis in Africans. *QJM* **91**:13–18.
- Bernig, T., J. G. Taylor, C. B. Foster, B. Staats, M. Yeager, and S. J. Chanock. 2004. Sequence analysis of the mannose-binding lectin (*MBL2*) gene reveals a high degree of heterozygosity with evidence of selection. *Genes Immun.* **5**:461–476.
- Boldt, A. B., A. Luty, M. P. Grobusch, K. Dietz, A. Dzeing, M. Kombila, P. G. Kremsner, and J. F. Kun. 2006. Association of a new mannose-binding lectin variant with severe malaria in Gabonese children. *Genes Immun.* **7**:393–440.
- Brabin, B. J. 1983. An analysis of malaria in pregnancy in Africa. *Bull. W. H. O.* **61**:1005–1016.
- Brabin, B. J., C. Romagosa, S. Abdelgalil, C. Menéndez, F. H. Verhoeff, R. McGready, K. A. Fletcher, S. Owens, U. D'Alessandro, F. Nosten, P. R. Fischer, and J. Ordi. 2004. The sick placenta—the role of malaria. *Placenta* **25**:359–378.
- Bulmer, J. N., F. N. Rasheed, N. Francis, L. Morrison, and B. M. Greenwood. 1993. Placental malaria. I. Pathological classification. *Histopathology* **22**:211–86174.
- Christiansen, O. B., D. C. Kilpatrick, V. Souter, K. Varming, S. Thiel, and J. C. Jensenius. 1999. Mannan-binding lectin deficiency is associated with unexplained recurrent miscarriage. *Scand. J. Immunol.* **49**:193–196.
- Fried, M., and P. E. Duffy. 1996. Adherence of *Plasmodium falciparum* to chondroitin sulfate A in the human placenta. *Science* **272**:1502–1504.
- Garred, P., M. A. Nielsen, J. A. L. Kurtzals, R. Malhotra, H. O. Madsen, B. Q. Goka, B. D. Akanmori, R. B. Sim, and L. Hviid. 2003. Mannose-Binding Lectin is a disease modifier in clinical malaria and may function as opsonin for *Plasmodium falciparum*-infected erythrocytes. *Infect. Immun.* **71**:5245–5253.
- Holmberg, V., F. Schuster, E. Dietz, J. C. Sagarriga Visconti, S. D. Anemana, U. Bienzie, and F. P. Mockenhaupt. 2008. Mannose-binding lectin variant associated with severe malaria in young African children. *Microbes Infect.* **10**:342–348.
- Ikeda, K., T. Sannoh, N. Kawasaki, T. Kawasaki, and I. Yamashina. 1987. Serum lectin with known structure activates complement through the classical pathway. *J. Biol. Chem.* **262**:7451–7454.

13. Kilpatrick, D. C., B. H. Bevan, and W. A. Liston. 1995. Association between mannan binding protein deficiency and recurrent miscarriage. *Hum. Reprod.* **10**:2501–2505.
14. Kilpatrick, D. C. 2000. Mannan-binding lectin concentration during normal human pregnancy. *Hum. Reprod.* **15**:941–943.
15. Klabunde, J., A. C. Uhlemann, A. E. Tebo, J. Kimmel, R. T. Schwarz, P. G. Kremsner, and J. F. Kun. 2002. Recognition of *Plasmodium falciparum* proteins by mannan-binding lectin, a component of the human innate immune system. *Parasitol. Res.* **88**:113–117.
16. Kruse, C., A. Rosgaard, R. Steffensen, K. Varming, J. C. Jensenius, and O. B. Christiansen. 2002. Low serum level of mannan-binding lectin is a determinant for pregnancy outcomes in women with recurrent spontaneous abortion. *Am. J. Obstet. Gynecol.* **187**:1313–1320.
17. Kuhlman, M., K. Joiner, and R. A. Ezekowitz. 1989. The human mannose-binding protein functions as an opsonin. *J. Exp. Med.* **169**:1733–1745.
18. Lipscombe, R. J., M. Sumiya, A. V. Hill, Y. L. Lau, R. J. Levinsky, J. A. Summerfield, and M. W. Turner. 1992. High frequencies in African and non-African populations of independent mutations in the mannose binding protein gene. *Mol. Genet.* **1**:709–715. (Erratum, **2**: 342, 1993.)
19. Luty, A. J., J. F. Kun, and P. G. Kremsner. 1998. Mannose-binding lectin and gene polymorphisms in *Plasmodium falciparum* malaria. *J. Infect. Dis.* **178**:1221–1224.
20. Madsen, H. O., P. Garred, J. A. Kurtzhals, L. U. Lamm, L. P. Ryder, S. Thiel, and A. Svejgaard. 1994. A new frequent allele is the missing link in the structural polymorphism of the human mannan-binding protein. *Immunogenetics* **40**:37–44.
21. Madsen, H. O., P. Garred, S. Thiel, J. A. Kurtzhals, L. U. Lamm, L. P. Ryder, and A. Svejgaard. 1995. Interplay between promoter and structural gene variants control basal serum level of mannan-binding protein. *J. Immunol.* **155**:3013–3020.
22. Madsen, H. O., M. L. Satz, B. Høgh, A. Svejgaard, and P. Garred. 1998. Different molecular events result in low protein levels of mannan-binding lectin in populations from southeast Africa and South America. *J. Immunol.* **161**:3169–3175.
23. Malhotra, R., A. C. Willis, A. Lopez Bernal, S. Thiel, and R. B. Sim. 1994. Mannan-binding protein levels in human amniotic fluid during gestation and its interaction with collectin receptor from amnion cells. *Immunology* **82**:439–444.
24. Manga, L., V. Robert, J. Mess, M. Desfontaine, and P. Carnevale. 1992. Le paludisme urbain à Yaoundé, Cameroun: l'étude entomologique dans deux quartiers centraux. *Mem. Soc. R. Belg. Entomol.* **35**:155–162.
25. McGregor, I. A. 1984. Epidemiology, malaria, and pregnancy. *Am. J. Trop. Med. Hyg.* **33**:517–525.
26. Mombo, L. E., C. Y. Lu, S. Ossari, I. Bedjabaga, L. Sica, R. Krishnamoorthy, and C. Lapoumeroulie. 2003. Mannose-binding lectin alleles in sub-Saharan Africans and relation with susceptibility to infections. *Genes Immun.* **4**:362–367.
27. Mombo, L. E., F. Ntoumi, C. Bisseye, S. Ossari, C. Yong Lu, R. L. Nagel, R. L., and R. Krishnamoorthy. 2003. Human genetic polymorphisms and asymptomatic *Plasmodium falciparum* malaria in Gabonese schoolchildren. *Am. J. Trop. Med. Hyg.* **68**:186–190.
28. Muehlenbachs, A., T. K. Mutabingwa, S. Edmonds, M. Fried, and P. E. Duffy. 2006. Hypertension and maternal-fetal conflict during placental malaria. *PLoS Med.* **3**:e446.
29. Muehlenbachs, A., M. Fried, J. Lachowitz, T. K. Mutabingwa, and P. E. Duffy. 2008. Natural selection of FLT1 alleles and their association with malaria resistance in utero. *Proc. Natl. Acad. Sci. USA* **105**:14488–14491.
30. Ordi, J., M. R. Ismail, P. J. Ventura, E. Kahigwa, R. Hirt, A. Cardesa, P. L. Alonso, and C. Menendez. 1998. Massive chronic intervillitis of the placenta associated with malaria infection. *Am. J. Surg. Pathol.* **22**:1006–1011.
31. Oudshoorn, A. M., F. A. van den Dungen, K. P. Bach, I. Koomen, W. P. Fetter, A. Catsburg, P. H. Savelkoul, and R. M. van Elburg. 2008. Mannose-binding lectin in term newborns and their mothers: genotypic and phenotypic relationship. *Hum. Immunol.* **69**:344–348.
32. Roestenberg, M., M. McCall, T. E. Mollnes, M. van Deuren, T. Sprong, I. Klasen, C. C. Hermesen, R. W. Sauerwein, and A. van der Ven. 2007. Complement activation in experimental human malaria infection. *Trans. R. Soc. Trop. Med. Hyg.* **101**:643–649.
33. Samuelson, J., S. Banerjee, P. Magnelli, D. J. Cui, J., Kelleher, R. Gilmore, and P. W. Robbins. 2005. The diversity of dolichol-linked precursors to Asn-linked glycans likely results from secondary loss of sets of glycosyltransferases. *Proc. Natl. Acad. Sci. USA* **102**:1548–1553.
34. Snounou, G., S. Viriyakosol, W. Jarra, S. Thaitong, and K. N. Brown. 1993. Identification of the four human malaria parasite species in field samples by the polymerase chain reaction and detection of a high prevalence of mixed infections. *Mol. Biochem. Parasitol.* **58**:283–292.
35. Steffensen, R., S. Thiel, K. Varming, C. Jersild, and J. C. Jensenius. 2000. Detection of structural gene mutations and promoter polymorphisms in the mannan-binding lectin (MBL) gene by polymerase chain reaction with sequence-specific primers. *J. Immunol. Methods* **241**:33–42.
36. Suguitan, A. L., Jr., R. G. Leke, G. Fouda, A. Zhou, L. Thuita, S. Metenou, J. Fogako, R. Megnekou, and D. W. Taylor. 2003. Changes in the levels of chemokines and cytokines in the placentas of women with *Plasmodium falciparum* malaria. *J. Infect. Dis.* **188**:1074–1082.
37. Sumiya, M., M. Super, P. Tabona, R. J. Levinsky, T. Arai, M. W. Turner, and J. A. Summerfield. 1991. Molecular basis of opsonic defect in immunodeficient children. *Lancet* **337**:1569–1570.
38. Tako, E. A., A. Zhou, J. Lohoue, R. Leke, D. W. Taylor, and R. F. Leke. 2005. Risk factors for placental malaria and its effect on pregnancy outcome in Yaounde, Cameroon. *Am. J. Trop. Med. Hyg.* **72**:236–242.
39. Thiel, S., U. Holmskov, L. Hviid, S. B. Laursen, and J. C. Jensenius. 1992. The concentration of the C-type lectin, mannan-binding protein, in human plasma increases during an acute phase response. *Clin. Exp. Immunol.* **90**:31–35.
40. van de Geijn, F. E., A. Roos, Y. A. de Man, J. D. Laman, C. J. de Groot, M. R. Daha, J. M. Hazes, and R. J. Dolhain. 2007. Mannose-binding lectin levels during pregnancy: a longitudinal study. *Hum. Reprod.* **22**:362–371.
41. van de Geijn, F. E., R. J. Dolhain, W. van Rijs, S. P. Willemsen, J. M. Hazes, C. J. de Groot. 2008. Mannose-binding lectin genotypes are associated with shorter gestational age. *Mol. Immunol. Hum. Immunol.* **68**:888–893.
42. Walter, P. R., Y. Garin, and P. Blot. 1982. Placental pathologic changes in malaria: a histologic and ultrastructural study. *Am. J. Pathol.* **109**:330–342.