Mutations of complement lectin pathway genes MBL2 and MASP2 associated with placental malaria

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Abstract

Background: Innate immunity plays a crucial role in the host defense against malaria including Plasmodium falciparum malaria in pregnancy, but the roles of the various underlying genes and mechanisms predisposing to the disease are poorly understood.

Methods: 98 single-nucleotide polymorphisms were genotyped in a set of 17 functionally related genes of the complement system in 145 primiparous Ghanaian women with placental malaria, defined by placental parasitaemia or malaria pigment, and as a control, in 124 non-affected primiparae.

Results: Placental malaria was significantly associated with SNPs in the lectin pathway genes MBL2, MASP2, FCN2 and in properdin. In particular, the main African mannose-binding lectin deficiency variant (MBL2*G57E, rs1800451) increased the odds of placental malaria (OR 1.6; permuted p-value 0.014). In contrast, a common MASP2 mutation (R439H, rs12085877), which reduces the activity of MBL-MASP2 complexes occurred in 33% of non-affected women and in 22% primiparae with placental malaria (OR 0.55, permuted p-value 0.020).

Conclusions: Excessive complement activation is of importance in the pathogenesis of placental malaria by mediating inflammation, coagulation, and endothelial dysfunction. Mutated MBL and MASP2 proteins could have direct intrinsic effects on the susceptibility to placental malaria, in addition to their roles in regulation of downstream complement activation.

Keywords: Lectin pathway, Mannose-binding lectin, MBL2, MASP2, Ficolin, Complement, Innate immunity, Malaria, Placenta, Pregnancy

Background

In sub-Saharan Africa, Plasmodium falciparum infection during pregnancy is a major cause of maternal anaemia, preterm delivery (PD), low birth weight (LBW) and infant mortality. In areas endemic for P. falciparum, 85 million pregnancies occur annually, and malaria-associated LBW in Africa results in an estimated 100,000 indirect infant deaths each year [1-3]. Pregnant, particularly primiparous, women are at increased risk. In pregnant women, parasites expressing specific variants of the P. falciparum Erythrocyte Membrane Protein-1 (PfEMP1) adhere to the placental endothelium lining the intervillous space which results in placental sequestration of infected red blood cells. The local accumulation of infected red blood cells and of malaria pigment (haemozoin), i.e. placental malaria, triggers the infiltration of inflammatory cells and a profound pro-inflammatory response [4,5]. This is confronted by an insufficient production of specific antibodies against the parasites and their PfEMP1 binding domain. Only with successive pregnancies, protective acquired immune mechanisms gradually develop and expand, and the disease manifestation declines [6-8]. In conditions of lacking or weak acquired immunity, e.g. early childhood and pregnancy, innate immune...
responses may play a predominant role in host defense against malaria [9]. Toll-like receptors (TLRs), for instance, are crucial mediators of innate immunity to *P. falciparum* [10], and variant signalling as deduced from functionally relevant single nucleotide polymorphisms (SNPs) has been associated with an increased risk and/or manifestation of malaria in pregnancy [11,12]. Overall, one third of the variability in susceptibility to placental malaria is thought to be due to host genetic factors [13]. These may also include the complement system, composed of three pathways, the classical, the lectin and the alternative pathway.

Mannose-binding lectin (MBL, encoded by *mbl2*) is a serum protein involved in the initiation of innate immune responses by binding to microbial surface oligosaccharides, and activating the lectin pathway. Upon binding, MBL forms a complex with mannanning-binding lectin serine peptidase 2 (MASP2) that cleaves C4 and C2 to form the C3 convertase (C4b2a). Subsequent complement activation leads to opsonization and phagocytosis of the target microbes, as well as formation of membrane attack complexes [14]. The role of MASP1 and MASP3 (encoded by *MASP1*) are still debated [15]. An important function of MASP1 is its ability to activate the alternative pathway proenzyme pro-D to active factor D [16]. MASP2 can also form active complexes with ficolins which bind to acetylated carbohydrates or, e.g., to acetylated LDL. Ficolins also recognize deposited C-reactive protein (CRP), and may thus collaborate with CRP in the initiation and control of inflammatory responses [15].

MBL binds to *P. falciparum*-infected red blood cells [17], and may consequently be involved in innate immune responses and parasite clearance. MBL deficiency caused by common SNPs increases the risk of severe malaria although findings are partially inconsistent [18-22]. As for (asymptomatic) placental infection, one recent study failed to show an association with MBL levels or *MBL2* genotypes [23].

The aim of the present study was to analyse the influence of lectin pathway polymorphisms on the susceptibility to placental malaria and its manifestation. Following a pathway-oriented approach and using Sequenom’s MassARRAY® system, SNPs of *MBL2*, *MASPs* and *ficolins* (FCN) and other important complement system genes (*SERPING1*, *C3*, *CFB*, *CFH*, *CFP*) were included in a novel “complement-chip”. Because the coagulation system may also be involved in adverse pregnancy outcomes [24], we also included respective regulatory genes (*thrombomodulin, thrombospordin, FLT1*).

Methods

Patients

A total of 893 women attending for delivery were recruited between January 2000 and January 2001 at the Presbyterian Mission Hospital in Agogo, located in the hyper-to holoendemic Ashanti Region of Ghana. The study protocol was reviewed and approved by the Committee on Human Research Publication and Ethics, School of Medical Sciences, University for Science and Technology, Kumasi, Ghana, and informed consent was obtained from all participants. Diagnostic procedures, malarialogic indices and clinical characteristics have been described earlier [11,24]. For the present study, all 304 primiparous women with live singleton deliveries were included. In brief, women were clinically examined, socioeconomic data were documented, and samples of placental intervillous and peripheral venous blood were collected into EDTA. Malaria parasites in intervillous blood were counted microscopically on Giemsa-stained thick blood films per 100 high-power fields (magnification x1,000), and the presence of leukocyte-associated haemoglobin was recorded. Placental infection was defined as the presence of *P. falciparum* parasites and/or haemoglobin in placental thick blood films. In addition, *P. falciparum*-specific PCR assays were performed [25]. Haemoglobin (Hb) was measured by a HemoCue photometer (Angelholm, Sweden), and anaemia defined as Hb < 110 g/l. Preterm delivery was defined as a gestational age < 37 weeks, on the basis of the Finnström score [26], and low birth-weight as < 2,500 g.

SNP selection

Based on our previous finding of an association of *MBL2* polymorphisms with severe malaria in children [22], all lectin pathway genes as well as other relevant downstream complement and alternative pathway genes were chosen for genotyping [27]. Additionally, we included genes related to the coagulation system (*thrombomodulin, thrombospordin*) and pre-eclampsia (*FLT1*). In total, 17 genes were chosen for genotyping (Table 1). For each gene, first, we selected SNPs with assumed relevance based on published data on protein function, activity or disease association. Secondly, we included potentially functional nonsense and missense SNPs, and finally, we also included some intronic, promoter or 3’ end SNPs as markers of association. We mainly focused on SNPs with minor allele frequencies > 0.05 in both African and European populations based on HapMap data.

Genotyping

DNA was extracted from peripheral blood (QIAmp; Qiagen, Germany). Twenty-seven of the samples from 304 primiparous women were excluded from genotyping based on low DNA quality or quantity. Among the 277 samples genotyped, 8 were removed because of a genotyping call rate < 0.9 leaving 269 samples for the final analyses.
SNP genotyping was performed using Sequenom’s MassARRAY MALDI-TOF Mass Spectrometry Compact platform and iPLEX Gold chemistry (Sequenom Inc, San Diego, CA) with standard protocols. A total of 98 SNPs were assayed in four multiplexes of 15 to 34 markers each. Eight SNPs that failed to meet a call rate of > 0.8 were removed. 14 SNPs (for which no previous frequency data were available) were excluded due to a minor allele frequency < 0.05, leaving 76 SNPs for the final analyses (Table 1). Genotypes were analysed using Sequenom’s MassARRAY Typer version 4.0 software. All data was checked twice manually and all outlying data or low intensity results were removed. The integrity of control and duplicate sample results, as well as negative control samples was checked during the evaluation process.

### Statistical analysis
Analyses were performed with PLINK v1.06 [28], SPSS (SPSS Inc, Chicago, Illinois; release 15.0, 2006) statistical software, and with R language. Hardy-Weinberg equilibrium was tested in infected and non-infected women separately, with the standard Chi-square test, to identify possible genotyping problems. Associations of placental malaria and the SNPs were tested with basic allelic association as well as P. falciparum positivity by microscopy or PCR from placental or peripheral blood samples. Permutation tests were used to adjust for multiple testing, as well as due to the correlatedness of the tests because of linkage disequilibrium (LD) between markers. Haplotypes were constructed and tested with PLINK using 2-to 6-marker sliding windows over all areas where adjacent SNPs were located less than 10 Mb from each other.

Odds ratios (OR) along with their confidence intervals (CIs) were estimated with PLINK and R scripts [29]. Joint ORs for two loci were evaluated by collapsing genotypes to susceptibility allele carriers and non-carriers, and combining these over both loci, resulting in just four susceptibility classes. This was done in order to avoid sparse class frequencies and thus overly wide confidence intervals.

Finally, interactions were screened with PLINK procedure epistasis, which tests all pairs of SNPs separated by at least 1 Mb and in different chromosomes. A $p$-value threshold of 0.001 was used to avoid most false positives. We acknowledge that our data is small for making robust inference on potential interactions, therefore these results should be considered preliminary.

### Results
The characteristics of the 269 primiparous Ghanaian women are shown in Table 2. Fifty-four percent of the women had placental malaria, defined as the presence of parasites or hemozoin in the intervillous placental blood (placental parasitemia, 48%, placental hemozoin, 43%).
Placental *P. falciparum*-PCR was positive in 66% and maternal peripheral blood *P. falciparum*-PCR positive in 59% of cases. Maternal hemoglobin and child birth weights were lower in cases with placental malaria than in uninfected mothers (*p* < 0.001 and *p* = 0.023). Gestational age did not differ between the groups.

A total of 76 SNPs of 17 candidate genes (Table 1) could be subjected to association analyses. SNPs with at least suggestive association with placental malaria were found on the **MBL2, MASP2, Ficolin 2 (FCN2)** and **Complement factor properdin (CFP)** genes (Table 3). Among these SNPs, **MBL2***C* (**G57E, rs1800451**), the main African MBL variant, which is associated with malaria in children [22], was present with an allele frequency of 36% in women with placental malaria, and 26% in controls (*p* = 0.01). The results suggested a dominant effect for an increased susceptibility to placental malaria (Table 3); homzygous or heterozygous **MBL2***C* was more frequently observed in women with placental malaria (61%, 88 out of 145) than in non-affected women (45%, 56 out of 123) resulting in an OR of 1.8 (95% CI 1.1-3.0) for the susceptbility allele in our data. **MBL***C* is significantly associated to microscopy positivity of placental blood samples, whereas other diagnostic tests show non-significant trends in the same direction (Table 4). **MBL2***C* is not associated to low birth weight, maternal anaemia or preterm birth.

With regard to **MASP2**, two markers, rs12085877 (**R439H, *p* = 0.02**) and rs1033638 (**3’ untranslated region, *p* = 0.03**), remained significantly associated with placental malaria after permutation tests. These exon 11 variants are located only 930 bp apart (Figure 1a). In this area, one major haplotype including both markers and spanning from rs2273347-rs2273346 conferred increased odds of placental malaria, with an overall *p* = 0.05, and for the haplotype AAGTA specifically, *p* = 0.021 (Table 5). However, the LD between the two major SNPs was surprisingly weak (rs12085877-rs1033638: *r*² = 0.037); and most of the risk increase could be attributed to R439H. This amino acid residue is located in the activation peptide of MASP2 (Figure 1b), between the second CCP domain and the serine protease domain, and the R439H mutation inhibits the normal function of the MBL-MASP2 complex [30]. When analysed with respect to different diagnostic tests, R439H showed strongest negative association to the presence of malaria pigment in the placenta (*p* = 0.008, OR 0.47, 95% CI 0.26-0.83), but was not associated to microscopy or PCR positivity of the placenta (Table 4). This would suggest a role of the association especially for chronic placental infection. Also, functionality of this mutation was supported by a non-significant trend towards low birth weight in individuals with the 439H variant (*p* = 0.057, OR = 0.51, 95% CI 0.24-0.99).

At the 5’ end of the **MASP2** gene, the region rs3765900-rs56392418-R99Q as a whole was associated with placental malaria at *p* = 0.05. Specifically, haplotype TGG was at present in 53% and 21% of women with and without placental malaria, respectively (*p* = 0.0051, Table 5; Figure 1a). None of the markers forming the haplotype were individually associated with placental malaria in permutation tests.

In the **FCN2** gene, two SNPs showed suggestive associations with placental malaria (Table 3). Located in...
intron 2 (rs3128624) and intron 3 (rs7037264), they are separated by only 73 bp. These SNPs were not associated to other phenotypes.

Complement factor properdin (CFP) is a positive regulator of the alternative complement pathway. It had one SNP associated with placental malaria (rs909523; allele C; \( p = 0.029 \) after permutation; Table 3). This variant, located in intron 6 of the gene, was not associated with any other assessed malaria phenotype. The region rs1048118-rs8177079-rs909523 was associated at \( p = 0.028 \), and specifically, the haplotype ATA was protective with \( p = 0.007 \) (present in 8% and 15% of women with and without placental malaria, respectively).

As MBL forms an active complex with MASP2, the potential interaction between the MBL2 and MASP2 genotypes was also analysed, to evaluate whether specific allele combinations of the two could render the individual susceptible to malaria. Placental malaria was detected in 39% of the women carrying no risk alleles for MBL2 (G57E) or MASP2 (R439 wildtype), compared to 65% of women with risk alleles at both loci (\( p = 0.007; \) OR 2.90, 95% CI 1.34-6.48). The increased risk for those with risk alleles at both loci compared to all other individuals gave an OR of 2.13 (95% CI 1.33-3.65, \( p = 0.003 \)). These results suggest an additive effect of both risk alleles, but an excess risk due to interaction could not be shown.

**Discussion**

Recent genome-wide association studies on malaria have had difficulties in identifying causal gene variants in placental malaria. The recent study by Holmberg et al. (2012) aimed to explore the associations of specific SNPs with placental malaria. The study utilized single nucleotide polymorphism (SNP) analysis to identify genetic variants associated with placental malaria. The authors found that SNP rs909523 in the CFP gene and the haplotype ATA were protective against placental malaria. Additionally, the study analyzed the interaction between MBL2 (G57E) and MASP2 (R439 wildtype) alleles, revealing an additive effect of both risk alleles but no evidence of an interaction effect.

**Table 3 Single marker results of allelic and genotypic association with placental malaria**

| Gene  | SNP      | Mutation | Allelic associations | Genotypic association |
|-------|----------|----------|----------------------|-----------------------|
|       |          |          | All. freq cases | All. freq controls | \( P \)-value | Permutated \( P \)-value | Odds ratio | 95% CI | Best genotypic model | Empirical \( p \)-value |
| MBL2  | rs1800451| G57E     | 0.36                | 0.26                | 0.014       | 0.014       | 1.80       | 1.10-2.32 | Dominant            | 0.021                  |
| MASP2 | rs12085877| H439R    | 0.11                | 0.19                | 0.014       | 0.020       | 0.55       | 0.34-0.89 | Allelic             | 0.015                  |
| MASP2 | rs1033638| 3’UTR    | 0.36                | 0.27                | 0.04        | 0.022       | 1.52       | 1.01-2.26 | Trend               | 0.064                  |
| FCN2  | rs3128624| Intron 2  | 0.67                | 0.62                | 0.198       | 0.204       | 1.27       | 0.88-1.81 | Dominant            | 0.053                  |
| FCN2  | rs7037264| Intron 3  | 0.68                | 0.64                | 0.271       | 0.261       | 1.23       | 0.85-1.76 | Dominant            | 0.042                  |
| CFP   | rs909523 | Intron 6  | 0.57                | 0.47                | 0.019       | 0.029       | 1.51       | 1.07-2.13 | Trend               | 0.043                  |

**Table 4 Association of different phenotypes of *P. falciparum* placental malaria with mutations MBL2*G57E and MASP2*R439H**

| MBL2*G57E (MBL2*C; rs1800451) | MASP2*R439H (rs12085877) |
|-----------------------------|---------------------------|
| **Wildtype (GG) Mutation (AA or AG) p-value OR** | **Wildtype (CC) Mutation (TT or TC) p-value OR** |
| Placental malaria | 45.5% (56/123) | 60.7% (88/145) | 0.014 | 1.84 (1.13-3.01) | 57.7% (113/196) | 43.8% (32/73) | 0.054 | 0.58 (0.33-0.99) |
| Maternal peripheral blood | | | | |
| PCR positive | 53.7% (66/123) | 63.4% (92/145) | 0.108 | 1.50 (0.92-2.45) | 62.2% (122/196) | 49.3% (36/73) | 0.070 | 0.59 (0.34-1.02) |
| Microscopy positive | 24.8% (29/117) | 29.0% (40/138) | 0.482 | 28.9% (55/190) | 22.7% (15/66) | 0.423 | | |
| Placental blood | | | | |
| PCR positive | 60.2% (74/123) | 70.3% (102/145) | 0.094 | 1.57 (0.94-2.61) | 68.4% (134/196) | 58.9% (43/73) | 0.151 | | |
| Microscopy positive | 39.0% (48/123) | 54.5% (79/145) | 0.014 | 1.86 (1.15-3.05) | 49.0% (96/196) | 41.1% (30/73) | 0.218 | | |
| Haemoglobin pigments present | 36.9% (45/122) | 48.3% (70/145) | 0.064 | 1.59 (0.98-2.62) | 48.0% (94/196) | 29.2% (21/72) | 0.008 | 0.47 (0.26-0.83) |
| Low birth weight | 27.0% (33/122) | 24.1% (35/145) | 0.673 | 28.6% (56/196) | 16.7% (12/72) | 0.057 | 0.51 (0.24-0.99) | |
| Maternal anaemia | 35.0% (43/123) | 40.7% (59/145) | 0.378 | 38.8% (76/196) | 35.6% (26/73) | 0.673 | | |
| Preterm birth | 27.0% (33/122) | 23.4% (34/145) | 0.571 | 23.5% (46/196) | 29.2% (21/72) | 0.344 | | |
African populations due to low LD [31,32]. In the present study, a pathway-oriented candidate gene approach to investigate the role of innate immunity and of the complement system in particular, deficiencies of which are known to increase susceptibility to a wide range of infections [33]. Genetic variants in the MBL2, MASP2, FCN2 and CFP genes were shown to be potential risk factors for placental malaria in Ghana. These results thus suggest a relevant role for the lectin pathway of the complement system in the human defense against placental malaria.

Functional MBL2 mutations interfere with the formation of oligomers and result in low serum levels of high molecular weight MBL and impaired MBL function [34]. Their role in malaria has been addressed in several studies [18-22,35], and a meta-analysis including four studies suggested an increased risk (odds ratio 1.29, 95% CI 1.08-1.53) for malaria in patients carrying the MBL2*C mutation [22]. The strongest association was found in young children [22] supporting the hypothesis that MBL is essential especially in individuals lacking fully developed semi-immunity. In a study from Mozambique, none of ten MBL2 SNPs examined were found to be associated with placental malaria [13], but MBL2*C was not included. The absence of association of the other SNPs can probably be explained by the low LD in Africans. These results suggest that the MBL2*C variant is a risk factor for placental malaria, in addition to infant malaria. It remains unclear, however, why such a disadvantageous disposition is maintained at a high frequency in malaria-endemic Africa; respective issues have been discussed elsewhere [21].

Protection from malaria provides an evolutionary benefit in Africa and respective SNPs can be expected to reach polymorphic frequencies. The MASP2 mutation R439H (rs12085877) is absent in European and Asian populations but according to a previous study [30] and HapMap data, its allele frequency is 9-15% in Sub-Saharan Africa [36]. In the present study, MASP2*R439H occurred in 27% of the women. In contrast to MBL2*C, it was protective against placent malaria, especially chronic infection, and a trend towards protection from

![Figure 1](a) The genetic map of the region of the MASP2 gene on chromosome 1p36.3-p36.2 is shown (a). The SNPs genotyped in this study are marked above the exons. (b) The polypeptide chain of MASP2 is composed of an N-terminal CUB domain, followed by an EGF domain, a second CUB domain, two CCP (complement-control protein) domains, an activation peptide, and a serine protease domain.

Table 5 Haplotype associations of MASP2 and CFP2 genes for placental malaria

| Gene area       | Overall p-value¹ | Specific haplotype | Prevalence in cases | Prevalence in controls | P-value²  |
|-----------------|------------------|--------------------|---------------------|------------------------|-----------|
| MASP2, exon 11  | rs2273347-rs2273346 0.05 | AAGTA              | 0.12                | 0.19                   | 0.021     |
| MASP2, 5' end   | rs3765900-rs6392418-R99Q 0.05 | TGG                | 0.53                | 0.21                   | 0.0051    |
| CFP             | rs1048118-rs8177079-rs909523 0.028 | ATA                | 0.08                | 0.15                   | 0.007     |

¹Overall p-value for association of the haplotypes in the gene area
²P-value for association of the specific haplotype
low birth weight was discernible. MASP2 with the R439H mutation is able to bind to MBL, but the enzymatic activity of the complex is considerably reduced [30]. At present, conclusive arguments are lacking for the differential impact on placental malaria of two genetic variants which finally lead to reduced complement activation. Thus, it could be possible that these mutations alter the direct intrinsic functions of MBL and MASP2 in addition to the downstream complement activation. MBL has been shown to stimulate phagocytosis and opsonization also in absent of other complement components [37]. MASP2 on the other hand, is capable of promoting fibrinogen turnover directly by cleavage of fibrinogen and indirectly by cleavage of prothrombin, generating active thrombin [38]. MASP2 can, in addition to MBL, interact with ficolins and the balance between formation of ficolin-MASP2 complexes and MBL-MASP2 complex could influence the susceptibility of placental malaria.

Another explanation for MASP2*R439H protecting against placental malaria could involve a curved production of C5a. Recent work has demonstrated that placental parasitaemia induces increased levels of this potent pro-inflammatory peptide in primiparae [39]. Data from adverse pregnancy outcomes in humans and from murine models of pathological pregnancies suggest that C5a could be an important regulator of placental angiogenesis, and excessive C5a could lead to functional placental insufficiency by impairing adequate vascularization of the placenta [40]. Elevated levels of C5a, however, are considered detrimental for host innate defense including defects in phagocyte and endothelial cell function [41]. Curved activation in this regard could thus be beneficial.

Alternatively, these findings on a common MASP2 variant show some resemblance with deficiency in the closely related complement receptor 1 (CR1). CR1 is also involved in the activation and regulation of C4, and patients with CR1 deficiency show greatly reduced rosetting, i.e. binding and aggregation of infected and non-infected erythrocytes [42]. Cytoadherence is the key feature of placental malaria [2,8], and small alterations of this process could have substantial clinical consequences. Increased generation of C4b and C3b would thus assist adherence and rosetting of malaria infected parasites in the placenta.

As for the ficolins, two SNPs on the FCN2 gene showed a suggestive association with placental malaria. These FCN2 variants are located in introns and are thus not likely to be functional themselves. On the contrary, they might be involved in gene expression regulation or splicing, or in LD with the actual predisposing mutations.

Downstream complement amplification of the lectin pathway is normally activated when MASP2 cleaves C4 and C2 to form the C3 convertase (C4b2a). C2 bypass of the lectin pathway is also possible, and involves alternative pathway amplification where complement factor properdin (CFP) and complement factor D (CFD) are involved [43]. This connection of CFP to the lectin pathway could be an explanation of its association to placental malaria. However, the downstream amplification and regulation of the lectin pathway is obviously complex and yet not fully understood.

Excessive complement activation has been suggested to play a role in the pathogenesis of severe malaria, including cerebral malaria as well as severe malarial anaemia, and placental malaria [27]. Through recognition of parasites by MBL and ficolins, the lectin pathway might be able to abort infections and reduce the parasitemic load, but on the other hand be harmful by mediating excessive inflammation, coagulation, and endothelial dysfunction. The novel finding of the protective role of the functional MASP2*R439H mutation makes MASP2 an interesting target for drug and vaccine development. MASP2 inhibitors need to be studied as potential adjunctive therapy for the various manifestations of malaria [44].
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doi:10.1186/1475-2875-11-61

Cite this article as: Holmberg et al: Mutations of complement lectin pathway genes *MBL2* and *MASP2* associated with placental malaria. *Malaria Journal* 2012 11:61.