MICROSCOPIC AND SUB-MICROSCOPIC PLASMODIUM FALCIPARUM INFECTION, BUT NOT INFLAMMATION CAUSED BY INFECTION, IS ASSOCIATED WITH LOW BIRTH WEIGHT

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Abstract. Pregnancy-associated malaria is one of the leading causes of low birth weight in malaria endemic areas. In this study, 145 parturient women residing in areas endemic for Plasmodium falciparum in Lambaréné, Gabon, were recruited into the study after delivery, and the association of maternal P. falciparum infection, inflammatory response, and birth weight was studied. At delivery, 10% (15) of the mothers (12 were positive in both peripheral and placental blood smears, 1 was positive in peripheral blood only, and 2 were positive in placenta blood only) were positive for P. falciparum by microscopy and 23% (30) by real-time polymerase chain reaction (PCR). The level of C-reactive protein (CRP) was significantly elevated in microscopically P. falciparum–positive pregnant women (34 mg/L; 95% CI: 3–458) but not in those with sub-microscopic infections (6 mg/L; 95% CI: 1–40) compared with those free of P. falciparum infection (7 mg/L; 95% CI: 1–43). In a multivariate analysis, the presence of microscopic (adjusted OR = 28.6, 95% CI = 4.8–169.0) or sub-microscopic (adjusted OR = 13.2, 95% CI = 2.4–73.0) P. falciparum infection in pregnant women and age of mothers < 21 years (adjusted OR = 9.7 CI = 1.0–89.7), but not CRP levels, were independent predictors for low birth weight. This finding may have important operational implications and emphasizes the need for appropriate diagnostic methods in studies evaluating the outcome of pregnancy-associated malaria.

INTRODUCTION

Pregnancy-associated Plasmodium falciparum malaria remains a major public health problem in endemic regions. In Africa > 24 million pregnancies are threatened every year by malaria.1 Pregnant women are at significantly greater risk of being infected with P. falciparum than non-pregnant women,2 and among pregnant women, P. falciparum infection is more often seen in pauciparous3,4 and in the youngest.4–6 Pregnancy-associated P. falciparum infection is one of the major causes of morbidity, leading to severe malaria, abortion, miscarriage, stillbirth in areas of low malaria endemicity, and mainly low birth weight in areas of intense malaria transmission.7–9 Low birth weight (< 2,500 g) constitutes the most important risk factor for infant mortality.10–12

The pathogenesis of low birth weight during malaria and pregnancy has been attributed to malaria-associated anemia in the mother13 and placental malaria, leading to prematurity and intrauterine growth retardation, accentuated in primigravid women.14,15 The parasite antigens expressed on the surface of infected erythrocytes bind to specific adhesion receptors in the placenta, especially chondroitin sulphate A, and result in parasite sequestration in the placenta.16,17 which is responsible for many of the harmful effects of malaria during pregnancy. P. falciparum infection has been shown to lead to inflammatory responses such as elevated T-helper 1 cytokines18,19 and increased levels of C-reactive protein (CRP),20,21 and both of these are associated with poor pregnancy outcome.22 However, many P. falciparum infections during pregnancy stay undetected when only microscopy of Giemsa-stained blood smears of peripheral blood but not placental malaria is used for diagnosis. These sub-microscopic infections can only be diagnosed by detection of circulating parasitic antigens or detection of parasite-specific DNA using conventional polymerase chain reaction (PCR), nested PCR,23–30 or the more recently developed real-time PCR.31–33 In previous studies, maternal sub-microscopic P. falciparum infection, as evaluated by nested PCR, was not significantly associated with adverse birth outcomes such as low birth weight.33,34 One recent paper has reported that quantitative PCR is superior to nested PCR in determining the burden of P. falciparum parasites.35 However, in this study, no comparison was made between microscopic and sub-microscopic P. falciparum infections. Here we used real-time PCR to evaluate the effect of sub-microscopic malaria on birth weight and also assessed the role that inflammation, as measured by elevated CRP plays in poor pregnancy outcome.

MATERIALS AND METHODS

Study area. The study took place in Lambaréné, Gabon, which is located amid the tropical rain forest of central Africa. Malaria is hyperendemic in this community with perennial transmission of P. falciparum malaria, and the entomologic inoculation rate (EIR) in this area has been estimated to average 50 infective bites per person per year.35,36 Screening for HIV is not routinely used in this setting. The prevalence of HIV in child-bearing women in Lambaréné has been estimated to be < 4%: data obtained from the mother-to-child HIV transmission clinic in Albert Schweitzer Hospital, a study conducted in 2003 during the study period (unpublished data).

Two mother–child health care centers that are part of the governmental hospital (General Hospital) and the private hospital (Albert Schweitzer Hospital) serve the local population.

Study population. Eligible women were recruited when reporting for delivery at the maternity clinic. Inclusion criteria were defined as 1) singleton fetal pregnancy, 2) residency in...
Lambaréné or the surrounding regions, and 3) written informed consent.

Exclusion criteria were defined as 1) refusal or withdrawal of consent and 2) serious illness.

After informed consent was obtained, data on demographic background and obstetrical history were recorded, and any missing data were completed by visiting the women after birth. Women were examined for vital signs.

The birth weight of the newborn was determined within 30 minutes after delivery using the mechanical baby scale SECA 725 Balance Beam (32 lb × 0.25 oz) (Gummersbach, Germany). Maternal peripheral blood was drawn within 30 minutes after delivery in an EDTA tube to determine the hemoglobin concentration, using the flow cytometry–based hematology analyzer (CellDyn 3000, Abbott Laboratories, Santa Clara, CA), and for CRP measurement (see below). The asexual *P. falciparum* parasites in maternal peripheral, cord, and placental blood obtained by aspiration between mother and cord interface was assessed by microscopy using the previously published method. Placental tissue and the pellet from mother’s peripheral blood obtained after centrifugation from the EDTA tube were collected and stored at −80°C for detection of *P. falciparum* DNA.

**DNA extraction.** DNA was extracted from 200 μL of EDTA blood pellet from the maternal peripheral blood and 50 mg of placental tissue, using the QIAamp DNA blood mini Kit (Qiagen, Hilden, Germany) according to the manufacturer’s instruction. In both cases, DNA was recovered in 200 μL of elution buffer from the isolation kit for use in PCR test to detect *P. falciparum*.

**Real-time PCR.** *P. falciparum*–specific PCR primers and a minor groove binding (MGB) Taqman probe were chosen using Primer Express software (Applied Biosystems, Foster City, CA) on the basis of the known SSU RNA gene sequence for *P. falciparum* (GenBank accession no. M19172), such that a 157-bp fragment within the SSU RNA gene should be amplified and detected specifically for *P. falciparum*. The *P. falciparum*–specific primers and probe set consisted of forward primer P-1047F 5′-GGTTTAGGAGGTGAAACGA-TCAGA-3′, reverse primer P-1178R 5′-AACCCAAAGAC-TTTGATTTCTCATAA-3′, and the *P. falciparum*–specific MGB Taqman probe P1-1141 VIC-5′-CTTTGAGGTGA0-CTTTTAGAT-3′-MGB non-fluorescent quencher (Applied Biosystems).

Amplification reactions were performed in a volume of 25 μL with PCR buffer (HotStarTaq mastermix; Qiagen), 5 mmol/L MgCl₂, 12.5 pmol of each *Plasmodium*-specific primer, 2.5 pmol of *P. falciparum*–specific MGB Taqman probe, and 5 μL of the DNA sample. Amplification consisted of 15 minutes at 95°C followed by 50 cycles of 15 seconds at 95°C and 60 seconds at 60°C. Amplification, detection, and data analysis were performed with the AB7500 real-time detection system (Applied Biosystems).

**CRP measurement.** Plasma was separated from maternal blood and umbilical venous blood (obtained from a clamped segment of the cord after birth and delivery of the placenta), collected into heparinized tubes and then centrifuged, and stored at −80°C. The concentration of CRP was detected with an immunoturbidimetric assay in a fully automated P-800 system (Hitachi, Tokyo, Japan). The interassay coefficients of variation (CVs) ranged from 1.8% to 2.5% at different levels, and the sensitivity was 0.5 mg/L.

**Definitions.** Primigravidae were those who were at their first pregnancy; multigravidae had multiple pregnancies.

Malarial infection status was defined as microscopic *P. falciparum* infection at delivery in peripheral blood or in placental blood smear. Sub-microscopic infection was defined as negative thick blood smear but positive for *P. falciparum* species with real-time PCR in peripheral blood and or in placenta tissue. The negative group was defined by the absence of *P. falciparum* as assessed by thick blood smear and by real-time PCR in peripheral blood and placenta tissue.

The ages were grouped into three categories: ≤ 21, between 21 and 27, and > 27 years of age.

The serum concentration of CRP is considered as evidence of inflammation, based on the current internationally accepted reference value of CRP is > 6 mg/L.

Low birth weight was defined as a newborn weight ≤ 2,500 g.

Anemia was defined if the hemoglobin value was < 11 g/dL and was considered normal if the hemoglobin rate was > 11 g/dL.

According to national guidelines at the time of the study (May 2003 until July 2004), all pregnant women were given chloroquine prophylaxis by the national malaria control program, despite a documented high-grade resistance of *P. falciparum* against this drug in this area. Iron and folic acid were given as well. The assessment of compliance with the prescribed regimen of chloroquine sulphate prophylaxis, iron, and folic acid was omitted because of the absence of active follow-up visits during pregnancy in this study.

**Ethical issue.** The ethics committee of the International Foundation of the Albert Schweitzer Hospital approved the study, and written informed consent was obtained from all participants or their parents in the case of minors.

**Statistics analysis.** Data were entered in File Maker Pro 5 (FileMaker, Santa Clara, CA) and transferred into SPSS for Windows 11.0 (SPSS, Chicago, IL) for statistical analysis. The age was grouped based on the 33rd and 66th percentile to provide equal numbers in the three groups.

For univariate analysis of categorical variables, Pearson’s χ² test was used. For continuous data, ANOVA t test was used for comparison between group means, except if the variances in the samples were not homogeneous. In this case, the non-parametric Kruskal-Wallis test was used. To estimate adjusted odds ratios (ORs) for low birth weight and inflammation, all factors associated with low birth weight in unadjusted analysis (P ≤ 0.05) were entered into the logistic regression model.

**RESULTS**

*P. falciparum* infection in maternal peripheral blood and in placental biopsies. As shown in Table 1, of the 145 pregnant women, *P. falciparum* infection was detected microscopically in 10% (15) of maternal peripheral blood and placental blood. Of these 15 samples positive by microscopy, 12 were positive both in placental and peripheral blood, 1 in peripheral blood only, and 2 in placental blood only.

From the remaining 130 pregnant women who were negative microscopically, 30 (23%) were positive for *P. falciparum* by real-time PCR and therefore carried sub-microscopic infection.

Three cord blood samples were positive microscopically for *P. falciparum*; these belonged to pregnant women who were
infected both peripherally and placentally. As expected, all microscopic malaria-positive samples were strongly positive in the PCR assay (data not shown).

**Levels of hemoglobin.** Hemoglobin (mean ± SD) levels were slightly lower in cases with a microscopically detectable *P. falciparum* infection (9.6 ± 1.7 g/dL) compared with hemoglobin levels in cases with a sub-microscopic infection (10.4 ± 1.3 g/dL), which were lower compared to controls (uninfected mothers; 10.7 ± 1.4 g/dL; *P* = 0.014).

**Levels of CRP in maternal and cord blood.** In women with a microscopically detectable *P. falciparum* infection, the levels of CRP (34 mg/L; 95% CI: 3–458) were significantly higher compared with CRP levels in women with a sub-microscopic *P. falciparum* infection (6 mg/L; 95% CI: 1–40; *P* < 0.0001) or CRP levels in non-infected women (7 mg/L; 95% CI: 1–43; *P* < 0.0001). When considering the levels of CRP > 6 mg/L, which indicates systemic inflammatory reaction, 47% of the pregnant women fell into this category, whereas in cord blood, three samples had levels of CRP > 6 mg/L (Table 1).

**Association between *P. falciparum* infection and birth weight.** Only 4% of the children born to non-infected women were of low birth weight. This is in contrast to the children delivered by women with a sub-microscopic *P. falciparum* infection, of which 23% (*P* = 0.001) had a low birth weight. Of children born to mothers with a microscopically detectable *P. falciparum* infection, 47% (*P* < 0.0001) were of low birth weight (Table 2). The mean weight of the children born to women with no *P. falciparum* infection was highest (3,103 ± 397 g), followed by lower birth weight (2,962 ± 473 g) in children born to mothers with sub-microscopic *P. falciparum* infection, whereas the lowest birth weight (2,806 ± 563 g) was in children born to mothers with microscopically detectable *P. falciparum* infection (*P* = 0.026).

**Association between CRP levels and birth weight.** The birth weight (mean ± SD) of newborns from mothers with CRP levels ≥ 6 mg/L was significantly lower (2,913 ± 230 g) compared with those from mothers with CRP levels < 6 mg/L (3,174 ± 394 g; *P* = 0.0001). Similarly, the proportion of children with a low birth weight born to women with CRP levels that marked systemic inflammation (> 6 mg/L) was significantly higher (17%) than in women who had no evidence of inflammation (6%, *P* = 0.03; Table 2).

**Risk factors for low birth weight.** Table 2 shows the risk factors for low birth weight as assessed in this study. In the univariate analysis, low birth weight was associated with microscopically *P. falciparum* infection (unadjusted OR = 21.0; 95% CI, 5.0–87.0; *P* = 0.0001), sub-microscopic *P. falciparum* infection (unadjusted OR = 7.3; 95% CI, 1.9–27.0; *P* = 0.003), and primigravidae (unadjusted OR = 3.1; 95% CI, 1.3–7.2; *P* = 0.008). Women < 21 years of age had a significantly increased risk for delivering neonates with low birth weight compared with women between 21 and 27 years of age (unadjusted OR = 15.9; 95% CI, 2.0–128.1; *P* = 0.019). Evidence of inflammation (CRP > 6 mg/L) increased the risk of low birth weight compared with the group with no sign of inflammation (unadjusted OR = 3.5; 95% CI, 1.0–11.7; *P* = 0.031). However, after adjusting in a multivariate analysis for *P. falciparum* infection status, parity, age group, and CRP levels ≥ 6 mg/L, it was found that the independent risk factors for low birth weight were the presence of microscopically *P. falciparum* parasite infection (adjusted OR = 28.6; 95% CI = 4.8–169.0), sub-microscopic *P. falciparum* parasite infection

<table>
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<tr>
<th>Table 1 Characteristics of maternal and neonatal study population</th>
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<td>Maternal age group</td>
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<td>21–27</td>
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<td>&gt; 27</td>
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<td>Parity</td>
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<td>Primiparous</td>
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<td>Hemoglobin (g/dL)</td>
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<td>&gt; 11</td>
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<td><em>P. falciparum</em> infection</td>
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<td>Total</td>
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<td>CRP level (mg/L)</td>
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<td>Neonate sex</td>
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<td>Female</td>
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<tr>
<td>Birth weight (g)</td>
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<tr>
<th>Table 2 Risk factors for low birth weight (newborn birth weight ≤ 2,500 g)</th>
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<td>Malaria Negative</td>
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<td>Sub-microscopic</td>
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<td>Microscopic</td>
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<td>Age group (years)</td>
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<td>&lt; 21</td>
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<td>≥ 27</td>
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<tr>
<td>Gravidity Primigravidae</td>
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<tr>
<td>Multigravidae</td>
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<tr>
<td>Inflammation No (CRP ≤ 6 mg/L)</td>
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<tr>
<td>Yes (CRP &gt; 6 mg/L)</td>
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Bold type indicates significant value (*P* < 0.05).
MICROSCOPIC AND SUB-MICROSCOPIC P. FALCIPARUM

(adjusted OR = 13.2; 95% CI = 2.4–73.3), and age < 21 years (adjusted OR = 9.7; 95% CI = 1.8–89.7) remained independent risk factors for low birth weight.

DISCUSSION

Pregnancy-associated malaria remains a major public health problem in endemic regions because of its detrimental effect on pregnancy outcome. It is important to assess the effects of different burdens of *P. falciparum* on pregnant women to determine accurately the morbidity caused by malaria in this population at risk. This cross-sectional study focused on microscopic and sub-microscopic *P. falciparum* infection and the presence of systemic inflammation at delivery and its implication on low birth weight.

Our major finding indicates that women with sub-microscopic levels of *P. falciparum* infection (detected by using the real-time PCR) at delivery had a 13-fold increased risk of delivering a child with low birth weight compared with non-infected pregnant women. Our results show that women with a microscopically detectable *P. falciparum* infection have a 2-fold increased risk for low birth weight compared with women with a sub-microscopic *P. falciparum* infection and 29-fold higher risk compared with non-infected women. The results in this study are consistent with the recent report on association between women with sub-microscopic *P. falciparum* infection using real-time PCR and the risk of having underweight offspring. However, their analysis was slightly different, in that although they have shown an association but did not assess sub-microbial infection as a risk factor using multivariate analysis, as done in this study. Conversely, these findings are in contrast with previous reports, where no statistically significant effect of sub-microscopic *P. falciparum* infection on low birth weight was observed.

Most studies investigating sub-microscopic *P. falciparum* infections have focused on its effect on maternal anemia or on systemic inflammation. Our results of assessing the risk factors for low birth weight are in accordance with findings from Cameroon with regard to the influence of age. However, in contrast to the above-mentioned study, we found no association between maternal anemia and low birth weight. Women participating in our study had higher hemoglobin levels compared with pregnant women in other malaria endemic regions, this might be because of public health measures including regular iron and folic acid supplements administered during antenatal visits, all of which may act as potential confounders in this study regarding the lack of association between anemia and birth weight. Indeed, this study shows a lower prevalence of *P. falciparum* (31%: 10% microscopic and 21% sub-microscopic) in pregnant women than reported in a previous study, in which 44% of pregnant women in the area of Lambarené were positive for sub-microscopic *P. falciparum* infection. Not only the difference in the extent of health care provided but also the difference in the diagnostic methods used might explain the discrepancies in the prevalence of sub-microscopic *P. falciparum* infections in pregnant women in the same area.

In this study, microscopic *P. falciparum* infection was associated with high CRP levels compared with those carrying sub-microscopic *P. falciparum* infection (*P < 0.0001*) and those free of detectable infection (*P < 0.0001*). This finding is in contrast to recent reports from Ghana where sub-microbial *P. falciparum* infections have been shown to lead to elevated CRP levels. This discrepancy might again be caused by the use of the different diagnostic methods and cut-off values used to categorize the infected and the uninfected groups. Furthermore, because of the anti-inflammatory effect of chloroquine, it could affect the CRP level found. However, the chloroquine level was not determined, and the prophylaxis dose might not sufficient to have an anti-inflammatory effect.

In agreement with the understanding that CRP is not produced by the fetal liver, no elevated levels of CRP were observed in cord blood samples, with the exception of three cases. These results indicate that CRP does not cross the placenta. In previous studies, elevated CRP levels found in amniotic fluid were associated with higher adverse pregnancy outcome reflect in preterm deliveries, intra-amniotic infection, chorioamnionitis, funisitis, and preeclampsia. In our study, we found that women with elevated CRP levels in plasma had a 3-fold increased risk for low birth weight compared with those who did not show signs of systemic inflammation; however, when adjusted for *P. falciparum* infection, no independent effect was observed. Taken together, *P. falciparum* (both microscopic and sub-microscopic) infection was the main cause of low birth weight in our study population.

However, we could not distinguish between premature delivery or in utero growth retardation as the cause of low birth weight.

Consistent with previous studies, it was found that even asymptomatic *P. falciparum* infections during pregnancy play an important role in the pathogenesis of low birth weight. It was further observed that only 3 of 15 (data not shown) microscopically positive women in this study were suffering from clinical malaria.

A potential limit of this study is the absence of data on HIV infection status, potentially confounding risk factor analysis for pregnancy adverse outcome including newborn weight, anemia in pregnancy, peripheral malaria, and placental malaria in our population. However, the HIV infection prevalence among pregnant women is estimated to be < 4% in Lambaréné (unpublished data).

Altogether, this study indicates that there is a need for highly sensitive and accurate diagnostic methods to study *P. falciparum* infections in pregnant women.

Currently intermittent presumptive treatment in pregnancy is recommended by the World Health Organization for areas of high malaria transmission. While this strategy seems desirable on an operational basis, further studies should elucidate whether sulfadoxine pyrimethamine (SP), the drug of choice, is efficacious enough to eliminate completely *P. falciparum* infections and therefore prevent adverse birth outcome caused by sub-microscopic parasitemia.

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