Submicroscopic *Plasmodium falciparum* Infections Are Associated With Maternal Anemia, Premature Births, and Low Birth Weight

Gilles Cottrell,1,2 Azizath Moussiliou,2,3 Adrian J. F. Luty,1,2 Michel Cot,1,2 Nadine Fievet,1,2 Achille Massougbodji,4 Philippe Deloron,1,2 and Nicaise Tuikue Ndam1,2

1PRES Sorbonne Paris Cité, Faculté de Pharmacie, Université Paris Descartes, 2Institut de Recherche pour le Développement, UMR216-MERIT, and 3Université Pierre et Marie Curie, Paris, France; and 4Centre d’Étude et de Recherche sur le paludisme associé à la Grossesse et à l’Enfance, Université d’Abomey-Calavi, Cotonou, Benin

**Background.** Molecular, as opposed to microscopic, detection measures the real prevalence of *Plasmodium falciparum* infections. Such occult infections are common during pregnancy but their impact on pregnancy outcomes is unclear. We performed a longitudinal study to describe that impact.

**Methods.** In a cohort of 1037 Beninese pregnant women, we used ultrasound to accurately estimate gestational ages. Infection with *P. falciparum*, hemoglobin concentration, use of intermittent preventive treatment during pregnancy (IPTp) for malaria, and other parameters were recorded during pregnancy. Using multivariate analyses, we evaluated the impact of submicroscopic infections on maternal anemia, premature birth, and low birth weight.

**Results.** At inclusion, polymerase chain reaction (PCR) and microscopy detected infection in 40% and 16% of women, respectively. The proportion infected declined markedly after 2 doses of IPTp but rebounded to 34% (by PCR) at delivery. Submicroscopic infections during pregnancy were associated with lower mean hemoglobin irrespective of gravidity, and with increased anemia risk in primigravidae (odds ratio [OR], 2.23; 95% confidence interval [CI], 0.98–5.07). Prospectively, submicroscopic infections at inclusion were associated with significantly increased risks of low birth weight in primigravidae (OR, 6.09; 95% CI, 1.16–31.95) and premature births in multigravidae (OR, 2.25; 95% CI, 1.13–4.46).

**Conclusions.** In this detailed longitudinal study, we document the deleterious impact of submicroscopic *P. falciparum* parasitemia during pregnancy on multiple pregnancy outcomes. Parasitemia occurs frequently during pregnancy, but routine microscopic and rapid diagnostic tests fail to detect the vast majority of episodes. Our findings imply caution in any revision of the current strategies for prevention of pregnancy-associated malaria.

**Keywords.** malaria; PCR; submicroscopic; pregnancy; *Plasmodium falciparum*.

Among malaria-causing parasites, *Plasmodium falciparum* is responsible for most morbidity and mortality, primarily during infancy and pregnancy in sub-Saharan Africa. Infected erythrocytes accumulating in placental intervillous spaces result in maternal anemia and low birth weight babies [1–4]. The impact of low birth weight on neonatal and infant morbidity and mortality is well known [5, 6]. Where malaria transmission is high, *P. falciparum* infection during pregnancy is more frequent in primigravidae, albeit commonly asymptomatic despite causing poor pregnancy outcomes [7, 8]. Malaria during pregnancy is responsible for up to 35% of preventable low birth weight deliveries, and contributes to 75 000–200 000 infant deaths annually [9].

Malaria diagnosis relies on examination of blood smears by microscopy, but rapid diagnostic tests (RDTs) are now in widespread use. Both techniques suffer from limitations in sensitivity. Polymerase chain reaction (PCR)–based molecular detection is
much more sensitive [10, 11], revealing up to 4-fold more infections than microscopy [12], challenging our view of what constitutes the reservoir of infection. Submicroscopic parasitemias have been described in multiple geographically separated regions [11, 13–15], but their clinical and public health relevance remains to be ascertained. In a pregnancy context, cross-sectional studies have reported the effects of submicroscopic infections on both maternal anemia and birth weight [10, 16–18], but their sample sizes were small and observations restricted to delivery. Submicroscopic infections of primigravid or secundigravid Malawian women [19] were related to placental infection at delivery, but not with adverse maternal or fetal outcomes, probably due to the very low proportion of submicroscopic infections in the study. Thus, detailed longitudinal studies investigating the effects of submicroscopic infections not only at delivery but also during pregnancy in women of all gravidities are lacking. Here, we present the results of a cohort study of 1037 pregnant women conducted in southern Benin. We analyzed the relationships between both submicroscopic and microscopic infections during pregnancy with pregnancy outcomes, including maternal anemia, premature birth, and birth weight.

METHODS

Ethics Statement
The Comité Consultatif de Déontologie et d’Ethique of the Research Institute for Development (France) and the ethics committee of the Faculty of Health Sciences (University of Abomey-Calavi, Benin) approved the study. All procedures complied with European and French national regulations.

Study Area
The study was conducted from May 2008 to May 2011 in Comé district, a semirural area of southern Benin, 70 km west of Cotonou, the commercial capital of Benin. Malaria transmission is perennial, with 2 peaks (April–July and September–November). The entomological inoculation rate is 35–60 [20], with *P. falciparum* predominating [21]. A detailed description of the study area was reported previously [22].

Study Design, Collection, and Handling of Blood Samples
The study comprised a prospective cohort of women recruited with gestational age (GA) <24 weeks in 3 maternity clinics: Comé central, Ouedeme-Pedah, and Akodeha. Women were followed up monthly from inclusion to delivery. Two doses of sulfadoxine-pyrimethamine (SP)–based intermittent preventive treatment during pregnancy (IPTp) were administered following national guidelines, directly observed by medical staff. At inclusion, at each antenatal visit, and at unscheduled “emergency” visits, when women presented at the clinic for health reasons, a RDT (Parascreen, Zephyr Biomedicals, Goa, India) for *P. falciparum* infection was performed on capillary blood, and venous blood was drawn. Hemoglobin (Hb) concentrations were determined using a HemoCue analyzer.

GA was estimated using transabdominal ultrasound. Four scans were performed with a portable ultrasonograph (Titan, Sonosite, Bothell, Washington) during follow-up by specifically trained midwives. As described [22], the first scan determined the exact GA, with those subsequently assessing intrauterine growth and fetal morphology. At delivery, placental and peripheral blood samples were collected. Thick and thin blood smears were prepared from all blood samples, stained with 10% Giemsa. *Plasmodium* parasites were counted against 200 leukocytes, allowing a detection threshold of 40 parasites/µL. Quality of slide reading was ensured through double reading by 2 experienced microscopists. In case of disagreement, a third reading was decisive. Four separate 50-µL drops of blood were spotted onto Whatman 3 filter paper, dried at room temperature, and stored with silica gel before subsequent DNA extraction using the Chelex method [23]. Data from blood samples obtained at inclusion, at second IPTp intake, 1 month before delivery, and at delivery were included in analyses.

Real-time PCR Assay for the Detection of *P. falciparum* Infections
The duplex real-time PCR assay used genus- and species-specific primers and probes for the small subunit (18S) gene of *Plasmodium* ribosomal RNA as reported [13]. Reaction mixtures contained 5 µL of DNA template (equivalent to 1.66 µL of whole blood), 10 µL of Master Mix (Applied Biosystem) containing both genus- and *P. falciparum*–specific primers and probes detection system (Plasmo/PF) in a final volume of 20 µL [13]. Samples underwent 40 cycles of amplification using the Viia 7 Real-time PCR system (Applied Biosystems). Quantification relied on a standard curve of DNA from cultured 3D7-strain *P. falciparum* (MR4, American Type Culture Collection). PCR-derived data came from samples at inclusion, second IPTp intake, 1 month before delivery, and at delivery.

Statistical Analysis
Of 1037 women enrolled, analyses were performed on 975 for whom GA, Hb level, and malarial infection status (diagnosed by blood smear and PCR) were available during the entire follow-up (Figure 1). Linear and logistic multivariate mixed models analyzed the relationships between malarial infection (submicroscopic or not), Hb level, and risk of anemia (defined as Hb <11 g/dL) during pregnancy, with adjustment for relevant covariates. To distinguish the proper effect of submicroscopic infection, a “3-class time-dependent infection” variable was built to characterize infections during the follow-up: At each visit, the status was “infected” if parasitemia was detected microscopically, “submicroscopically infected” if the thick blood smear
was negative and the PCR was positive, and “negative” if both thick blood smear and PCR were negative.

In these analyses, adjustment factors were gravidity (primigravidae/multigravidae), body mass index (at inclusion), season at the current visit (4 classes: beginning/end of rainy season, beginning/end of dry season), intake of antimalarial drugs (other than SP-IPTp) during pregnancy, total number of malarial infections detected by microscopy during pregnancy, and GA at the current visit.

Relationships between birth weight, occurrence of low birth weight (<2500 g), premature birth (defined as birth before GA 37 weeks), and malarial infection (submicroscopic and microscopic) at inclusion were assessed through linear (and logistic) multivariate regression. Twins, abortions, and stillbirths were excluded from premature birth and birth weight analyses. Premature babies were excluded from birth weight analyses (Figure 1). Here, adjustment factors were gravidity (primigravidae/multigravidae), body mass index (at inclusion), season (4 classes: beginning and end of rainy season, beginning and end of dry season), intake of antimalarial drugs (other than SP-IPTp) during pregnancy, number of malarial infections during pregnancy, and (only in birth weight analyses) GA at delivery.

Analyses were done first using the complete sample of women, then analyses were stratified on gravidity to study its interaction with malarial infection status.

RESULTS

Mean GA at inclusion was 16.5 weeks (standard deviation [SD], 4.8 weeks), and 18.5% of women were primigravidae. Mean Hb level at inclusion was 10.6 g/dL (SD, 1.2 g/dL; range, 5.1–14.4 g/dL), and 61.1% of women presented with anemia. Mean birth weight was 2978 g (SD, 503 g) in newborns with available birth weight, and 3055 g (SD, 426 g) excluding those born prematurely, twins, abortions, and stillbirths. Among 873 women with an estimate of GA, 9.9% presented with preterm deliveries and 7.5% with twins, abortions, or stillbirths.

Mean number of complete visits (with thick blood smear, PCR, and Hb level) per woman was 3.0 (range, 1–4), and 72.4% of the women had 3 or 4 complete visits during the follow-up. Mean number of ultrasound scans per woman during the follow-up was 3.5 (range, 1–4).

Figure 2 shows microscopic and submicroscopic infection profiles during follow-up according to gravidity. The cumulated prevalence at inclusion (microscopic and submicroscopic infections combined) was 39.7%, almost two-thirds of which were submicroscopic. The prevalence decreased to 9.3% at the time of second IPT administration, showing the effect of the first dose of IPT, but subsequently increased to 34.2% at delivery. The shapes of the malarial infection dynamics were quite similar between primigravidae and multigravidae, with some expected differences: Primigravidae were more often infected at inclusion than multigravidae (51.7% vs 37.1%; P < .001); microscopic infections predominated in primigravidae (59.5% of total infections); and submicroscopic infections predominated (64.2% of total infections) in multigravidae.

Table 1 shows the association between Hb level and malarial infection at the time of detection. Submicroscopic infections were associated with decreased mean Hb level during pregnancy in all women, as well as separately in both primigravidae and multigravidae in stratified analyses, the effect being more pronounced in primigravidae. As expected, microscopically detectable infections in both primigravidae and multigravidae were associated with stronger reductions in mean Hb levels compared with submicroscopic infections.

No association was observed between submicroscopic infection and risk of anemia in the whole group (Table 2), but it was of borderline significance for primigravidae. The high odds ratio (a >2-fold higher risk of anemia in women with submicroscopic infections) suggests that strict significance would have been reached with a larger sample size. No such association for submicroscopic infections was observed in multigravidae. Women with microscopic infections had a higher risk of anemia compared with uninfected women, regardless of gravidity.

Microscopic infection at inclusion was related to decreased mean birth weight with a borderline significance (P = .067; Table 3). This is consistent with previous reports on the same
data and in Burkina Faso reporting decreased mean birth weight as a function of infections in early pregnancy (months 0–4) [22, 24]. Also as expected, in the overall multivariate analysis, newborns of primigravidae had a lower mean birth weight (−132.7 g; \( P < .001 \)) as well as an increased risk of low birth weight (odds ratio, 2.24; \( P = .017 \)) compared with those of multigravidae. The stratified analysis revealed that in multigravidae, microscopic infections at inclusion were related to decreased mean birth weight, whereas submicroscopic infections were not, and infections of neither type influenced the risk of low birth weight (Table 4). In primigravidae, mean birth weight was unaffected by infections of either type, but there was a higher risk of low birth weight in those with submicroscopic infections at inclusion (odds ratio, 2.24; \( P = .017 \)).

### Table 1. Relationship Between *Plasmodium falciparum* Infection at the Time of Detection and Hemoglobin Level During Pregnancy, Multivariate Linear Mixed Model (n = 975)

<table>
<thead>
<tr>
<th>Type of Infection</th>
<th>Mean Hemoglobin Differencea, g/dL</th>
<th>( P ) Value</th>
<th>95% Confidence Interval</th>
</tr>
</thead>
<tbody>
<tr>
<td><strong>Submicroscopic infection</strong></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Overall sample</td>
<td>−0.16</td>
<td>.001</td>
<td>−.25 to −.06</td>
</tr>
<tr>
<td>Primigravidae</td>
<td>−0.47</td>
<td>&lt;.001</td>
<td>−.72 to −.21</td>
</tr>
<tr>
<td>Multigravidae</td>
<td>−0.10</td>
<td>.052</td>
<td>−.20 to .00</td>
</tr>
<tr>
<td><strong>Microscopic infection</strong></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Overall sample</td>
<td>−0.51</td>
<td>&lt;.001</td>
<td>−.63 to −.39</td>
</tr>
<tr>
<td>Primigravidae</td>
<td>−0.74</td>
<td>&lt;.001</td>
<td>−.99 to −.49</td>
</tr>
<tr>
<td>Multigravidae</td>
<td>0.44</td>
<td>&lt;.001</td>
<td>−.58 to −.29</td>
</tr>
</tbody>
</table>

a Difference between infected and noninfected women (reference class) adjusted for body mass index (at inclusion), season, intake of malaria treatment (other than intermittent preventive treatment) during pregnancy, total number of malaria infections during pregnancy, gestational age, and (for overall sample analyses) gravidity.

b Primigravidae: \( n = 179 \); multigravidae \( n = 796 \).

### Table 2. Relationship Between *Plasmodium falciparum* Infection at the Time of Detection and Anemiaa During Pregnancy, Multivariate Mixed Model (n = 975)

<table>
<thead>
<tr>
<th>Type of Infection</th>
<th>Odds Ratiob</th>
<th>( P ) Value</th>
<th>95% Confidence Interval</th>
</tr>
</thead>
<tbody>
<tr>
<td><strong>Submicroscopic infection</strong></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Overall sample</td>
<td>1.09</td>
<td>.57</td>
<td>.80–1.49</td>
</tr>
<tr>
<td>Primigravidae</td>
<td>2.23</td>
<td>.056</td>
<td>.98–5.07</td>
</tr>
<tr>
<td>Multigravidae</td>
<td>0.97</td>
<td>.88</td>
<td>.69–1.38</td>
</tr>
<tr>
<td><strong>Microscopic infection</strong></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Overall sample</td>
<td>3.86</td>
<td>&lt;.001</td>
<td>2.47–6.02</td>
</tr>
<tr>
<td>Primigravidae</td>
<td>7.03</td>
<td>&lt;.001</td>
<td>2.59–19.11</td>
</tr>
<tr>
<td>Multigravidae</td>
<td>3.39</td>
<td>&lt;.001</td>
<td>2.02–5.71</td>
</tr>
</tbody>
</table>

a Defined as a hemoglobin level <11 g/dL.

b Odds ratio of the risk of anemia in infected vs noninfected women (reference class) adjusted for body mass index (at inclusion), season, intake of malaria treatment (other than intermittent preventive treatment) during pregnancy, total number of malaria infections during pregnancy, gestational age, and (for overall sample analyses) gravidity.

c Primigravidae: \( n = 179 \); multigravidae \( n = 796 \).
infections (odds ratio, 6.09; P = .033) than in those with microscopic infections compared to negative (P = .13).

Both submicroscopic and microscopic infections were associated with premature births in multigravidae, with a >2-fold increased risk in infected women (Table 5), but not in primigravidae.

**DISCUSSION**

To our knowledge, this is the first large cohort study of women of all gravidities exposed to malaria investigating the effects of *P. falciparum* submicroscopic (as well as microscopic) infections on various pregnancy outcomes. The incorporation of ultrasound further distinguishes it from foregoing studies, along with PCR-based detection of low-density infections. Our findings clearly demonstrate the importance of submicroscopic infections during pregnancy in public health terms, especially in primigravidae, revealing their associations with maternal anemia, premature births, and low birth weight.

The high proportion of submicroscopic infections detected both at inclusion, before the first dose of SP-IPTp (55% received their first dose at a visit subsequent to inclusion), and at delivery, is consistent with other studies, confirming that the parasite reservoir is much greater than that predicted by microscopy [25, 26]. The first dose of SP-IPTp was given relatively early in pregnancy (median GA, 21 weeks), the second, 1 month later, occurred at 25.5 weeks. This early SP-IPTp administration shifts the coverage window, likely explaining why the proportion infected increased dramatically (34.2%) at delivery. Thus, even women receiving 2 doses of SP-IPTp under direct observation, and subject to close follow-up, were not adequately protected against *P. falciparum* infection in the third trimester of pregnancy. The infection dynamics also reflect the high rates of recurrent infections after the second SP-IPTp dose, indicating the degree of SP resistance in Benin [27]. At inclusion, the submicroscopic to microscopic infection ratio in multigravidae was higher than in primigravidae. This accords well with other observations in comparable transmission settings, and is likely attributable to stronger acquired immunity of multigravidae [16].

Previous studies conducted at delivery reported the relationships between submicroscopic infections and Hb level or risk of anemia [10, 16, 26]. Decreased Hb levels associated with submicroscopic infections have been described in primigravidae and...
Our study is the first to show that submicroscopic infections during pregnancy also increase the risk of anemia in primigravidae, echoing earlier observations at delivery [16]. As expected [28–30], microscopic infections were associated with lower Hb levels and increased risks of anemia in both primigravidae and multigravidae.

We know of only 2 studies reporting associations between submicroscopic infections and low birth weight. Both were cross-sectional, conducted at delivery, in Gabon where transmission is stable and perennial, as in Benin, and in Sudan where transmission is unstable [17, 18]. In our setting, submicroscopic infections at delivery were not associated with birth weight (data not shown), likely the consequence of the close follow-up of the women, of the SP-IPTp administered, and of the antimalarial treatment given whenever parasitemia was detected. As a result, and as our genotypic analyses show (unpublished data), the majority of infections at delivery were recently acquired, having not been established long enough to have significant effects. In the Malawian study [19], submicroscopic infections and birth weight were not associated, probably due to a lack of power in the analyses, something the authors acknowledged. Here, submicroscopic but not microscopic infections at inclusion (ie, early in pregnancy) were associated with an increased risk of low birth weight in primigravidae. This differential impact on outcomes may reflect the fact that the microscopic infections were immediately treated, whereas submicroscopic infections were not for most women (only 38% of primigravidae presenting with submicroscopic infection received their first SP-IPTp dose at inclusion), with a consequent potential for persistence and chronicity. Infections early in pregnancy can cause serious adverse pregnancy outcomes, especially low birth weight [22, 24], and, importantly, placental-type parasites are present even in the first trimester of pregnancy [31].

In multigravidae but not in primigravidae, we observed prospective associations (1) between microscopic infection at inclusion and significantly lower mean birth weight, and, intriguingly, (2) between submicroscopic infections and a significantly increased risk of premature birth. We conjecture in this context that both lower mean birth weight and premature birth in multigravidae have a common causality that reflects their more proinflammatory interferon γ–biased acquired immune response to “placental-type” P. falciparum infections compared to primigravidae [32]. Plausibly, then, treatment of microscopic infections at inclusion in multigravidae would be expected to exacerbate any such preexisting proinflammatory activity. Equally, persisting untreated submicroscopic infections in multigravidae would allow proinflammatory responses themselves to persist, the important point being that inflammatory responses per se are known to be associated with poor pregnancy outcomes, including premature birth and low birth weight. Whatever the cause, these novel findings certainly merit further exploration.

CONCLUSIONS

This study, the first to our knowledge to analyze submicroscopic infections with P. falciparum during pregnancy in women of all gravidities, reveals their associations with poor pregnancy outcomes. The large sample size compared with previous studies, as well as the close follow-up and the use of ultrasound to accurately determine GAs, all contributed to enhancing the quality...
and depth of our analyses. All of these elements combined lead to novel findings concerning the impact of submicroscopic infections with *Plasmodium falciparum* on maternal (anemia) and fetal (premature birth, birth weight) health-related parameters. Primigravidae are considered to be most susceptible to placental malaria and its consequences. Our data serve to reassert that precept, while further extending it to the context of submicroscopic infections. The prospective analyses highlighted the fact, yet again, that *P. falciparum* infection early in pregnancy, when women do not habitually benefit from antimalarial preventive measures, leads to poor birth outcomes. Overall, the results challenge fundamentally the current view of malaria epidemiology and the burden of infection during pregnancy, and confirm the urgent need to (1) further evaluate the importance of submicroscopic infections as a reservoir for transmission in pregnant women, (2) determine the importance of the timing and frequency of submicroscopic infections during pregnancy in terms of key public health outcome measures, and (3) intensify the research for and development of molecular diagnostic tools for field use to complement or to replace those currently used that lack sufficient sensitivity.

**Notes**

**Acknowledgments.** We are grateful to all the women who participated in the study. We thank all the medical staffs of Akodeha, Come Central, and Ouedeme Pedah health centers for their valuable contributions.

**Author contributions.** N. T. N., G. C., and P. D. conceived and designed the study. A. Mo. and N. T. N. performed the laboratory experiments. G. C. and N. T. N. analyzed the data. N. F., A. Ma., and M. C. contributed reagents/materials/analysis tools. G. C., A. J. F. L., P. D., and N. T. N. drafted and N. T. N. analyzed the data. N. F., A. Ma., and M. C. contributed reagents/materials/analysis tools. G. C., A. J. F. L., P. D., and N. T. N. drafted and finalized the manuscript. The final manuscript was read and approved by all authors.

**Disclaimer.** The funders had no role in study design, data collection and analysis, decision to publish, or preparation of the manuscript.

**Financial support.** This article describes work undertaken in the context of the STOPPAM (Strategies to Prevent Pregnancy Associated Malaria) and PLACMALVAC (Clinical Development of a VAR2CSA-Based Placental Malaria Vaccine) projects. STOPPAM and PLACMALVAC are small-scale collaborative projects supported by the European 7th Framework Programme (contract numbers 200889 and 304815). A. Mo. was supported by PhD studentships from the Islamic Development Bank.

**Potential conflicts of interest.** All authors: No potential conflicts.

All authors have submitted the ICMJE Form for Disclosure of Potential Conflicts of Interest. Conflicts that the editors consider relevant to the content of the manuscript have been disclosed.

**References**