Clinical Infectious Diseases

MAJOR ARTICLE

Persistent *Plasmodium falciparum* Infection in Women With an Intent to Become Pregnant as a Risk Factor for Pregnancy-associated Malaria

Nicaise Tuikue Ndam,[1,2] Bernard Tornyigah,[1,2] Akpéyédjé Yannelle Dossou,[3] Guillaume Escriou,[1] Morten A. Nielsen,[4,5] Ali Salanti,[4,5] Saadou Issifou,[3] Achille Massougbodji,[3] Jean-Philippe Chippaux,[1,3] and Philippe Deloron[1]

[1]Mère et Enfant face aux Infections Tropicales, Institut de Recherche pour le Développement, Université Paris 5, Sorbonne Paris Cité, France; [2]Department of Parasitology, Noguchi Memorial Institute for Medical Research, College of Health Sciences, University of Ghana, Legon; [3]Centre de Recherche sur le Paludisme Associé à la Grossesse et l'Enfance, Cotonou, Benin; and [4]Centre for Medical Parasitology at Department of Immunology and Microbiology, Faculty of Health and Medical Science, University of Copenhagen, and [5]Department of Infectious Diseases, Copenhagen University Hospital (Rigshospitalet), Denmark

Background. Pregnant women are more susceptible to *Plasmodium falciparum* than before pregnancy, and infection has consequences for both mother and offspring. The World Health Organization recommends that pregnant woman in areas of transmission receive intermittent preventive treatment (IPTp) starting in the second trimester. Consequently, women are not protected during the first trimester, although *P. falciparum* infections are both frequent and harmful.

Methods. A cohort of nulligravid women was followed up during subsequent pregnancy. Malaria was diagnosed by means of microscopy and polymerase chain reaction. Parasites were genotyped at polymorphic loci.

Results. Among 275 nulligravidae enrolled, 68 women became pregnant and were followed up during pregnancy. Before pregnancy, *P. falciparum* prevalence rates were 15% by microscopy and 66% by polymerase chain reaction. Microscopic infection rates increased to 29% until IPTp administration, and their density increased by 20-fold. Conversely, submicroscopic infection rates decreased. After IPTp administration, all types of infections decreased, but they increased again late in pregnancy. The risk of infection during pregnancy was higher in women with a microscopic (odds ratio, 6.5; $P = .047$) or submicroscopic (3.06; $P = .05$) infection before pregnancy and was not related to the season of occurrence. Most infections during pregnancy were persistent infections acquired before pregnancy.

Conclusions. Microscopic and submicroscopic malaria infections were frequent in nulligravid women from south Benin. During the first trimester of pregnancy, microscopic infections were more frequent, with a higher parasite density, and mainly derived from parasites infecting the woman before conception. Preventive strategies targeting nonpregnant women with a desire for conception need to be designed.

Keywords. malaria; pregnancy; genotypes; preconception.

Despite huge achievements in malaria control programs, *Plasmodium falciparum* malaria remains a major public health problem in most tropical endemic areas. Pregnant women are particularly concerned, because they become more susceptible to *P. falciparum* infection than before their pregnancy, and because the infection affects both mother and child. Therefore, the World Health Organization (WHO) recommends that pregnant women in areas of moderate to high malaria transmission receive intermittent preventive treatment (IPTp). IPTp is given as a full therapeutic course of sulfadoxine-pyrimethamine (SP) at each scheduled antenatal care visit, starting as early as possible in the second trimester [1]. Consequently, IPTp cannot protect women during the first trimester of pregnancy, although *P. falciparum* infections are then both frequent and harmful [2].

When a woman exposed to or carrying *P. falciparum* parasites, even asymptomatically, becomes pregnant, the presence of a placenta provides a novel binding partner for parasite populations that otherwise would be cleared by the spleen. A vaccine dedicated to preventing pregnancy-associated malaria would, therefore, be highly valuable in this context. Such a vaccine to be administered before women become pregnant is currently in the early phase of clinical development [3, 4], and hence not yet available. Identification of infected women during early pregnancy, and a better understanding of the risk factors associated with such early infections may allow their best management by appropriate intervention strategies [5].

In peripheral health centers, malaria diagnosis relies on the rapid diagnostic test or thick blood smear, neither of which is highly sensitive. Previous reports indicated that even infections with very low parasite densities in the peripheral blood, so-called submicroscopic infections, are associated with poor

Received 5 March 2018; editorial decision 18 April 2018; accepted 3 May 2018; Published online May 4, 2018.

Correspondence: N. Tuikue Ndam, MERIT, IRD, 4 Avenue de l'Observatoire, 75006 Paris, France (nicaise.ndam@ird.fr).

Clinical Infectious Diseases® 2018;67(12):1890–6

DOI: 10.1093/cid/ciy380

pregnancy outcome with an impact on anemia, prematurity and low birth weight of the offspring in Benin [6], whereas infections detected by means of polymerase chain reaction (PCR) but not with rapid diagnostic tests were not associated with poor outcomes in infants in Malawi, Ghana, or India [7].

In the current study, we enrolled nonpregnant women of childbearing age and followed them up during their subsequent first pregnancy. Using highly sensitive PCR techniques, we show that women infected with malaria while not pregnant, even with submicroscopic infections, were more likely to present with a *P. falciparum* infection when they become pregnant. Most of these infections during pregnancy resulted from the persistence of the infection acquired before pregnancy.

METHODS

Study Population and Design

The study was conducted in the district of Sô-Ava, 25 km North of Cotonou, Benin. This lagoon area is characterized by a subtropical climate, with 2 rainy seasons (April–July and October–November) and 2 dry seasons. Malaria is hyperendemic, with a mean entomological inoculation rate of 2.05 (standard deviation, 1.3) infective bites per person per 100 nights [8]. Nulligravid nonpregnant women with a desire to become pregnant were enrolled from February 2014 and followed up through conception and pregnancy to delivery. The follow-up ended in February 2016 after the birth of the last newborn. Initially, we aimed to enroll 600 young nulligravid women in a 24-month primary cohort (cohort 1) to identify 50 women who become pregnant and enter a secondary cohort (cohort 2). After enrollment in the primary cohort, women were visited monthly at home to record the first day of last menstrual period, and to perform a urinary pregnancy test (One-Step; International Holding Corp). When the follow-up of the primary cohort ended, nonpregnant women were offered the opportunity to consult a gynecologist for fertility status assessment. All women recruited lived in the Sô-Ava Commune and were of the "Toffin" ethnic group.

At enrollment in the primary cohort, demographic and socioeconomic characteristics were collected. Malaria screening was performed. Once women entered the secondary cohort, they were seen monthly at each scheduled antenatal care visit until delivery and at a final visit 6 months after delivery. Pregnant women were managed according to Benin Ministry of Health guidelines, with supply at the first antenatal care visit of a kit including iron and folic acid, mebendazole for deworming, a long-lasting insecticide-treated net, and the first dose of SP for IPTp to take in the second trimester. Although mosquito nets were distributed to all pregnant women, we could not verify their use, but the strong mosquito nuisance on the lagoon might account for high bed net usage. Maternity staff were encouraged to administer ≥3 doses of IPTp with SP (IPTp-SP) from the second trimester onward. At delivery, newborn characteristics were collected. Maternal capillary and placental blood samples were collected. At each visit, 50 µL of blood was transferred to Whatman 3MM filter paper for DNA extraction. *Plasmodium falciparum* infections were confirmed by microscopic examination of Giemsa-stained thick blood smears, and parasitemia was recorded as the number of asexual parasites per microliter of blood.

Ethics Statement

Ethical clearance was obtained from the Institutional Ethics Committee of the Faculty of Health Science, Abomey-Calavi University (No. 12/09/2013/CE/FSS/UAC) and the Comité Consultatif de Déontologie et d'Ethique from Institut de Recherche pour le Développement. Written informed consent was obtained from each woman.

Parasitological Determinations by Microscopy and Real-time PCR

Thick and thin blood films were read for *Plasmodium* species according to standard, quality-controlled procedures [9]. The presence of *P. falciparum* was also tested in duplicate using real-time quantitative PCR that targeted the 18S ribosomal DNA gene [9, 10]. Samples without amplification after 40 cycles of amplification were considered negative. Parasitemia was quantified by extrapolation of cycle thresholds from a standard curve of DNA from 3D7 *P. falciparum* ring stage–infected erythrocyte culture. A negative control with no DNA template was assayed in all reactions. *Plasmodium falciparum* infections detected with quantitative PCR, but not with microscopy, were considered submicroscopic [6].

Molecular Genotyping of the Polymorphic Genes *msp1* (Block 2) and *msp2*

Analysis of *msp2* (3D7 and FC27 allelic families) and *msp1* block 2 (K1, MAD20, and RO33 allelic families) of *P. falciparum* were performed by nested PCR, as described by Snounou et al [11]. The study was performed on infected samples collected at enrollment in cohort 1 (before pregnancy), and then in the same women at recurrent infections during pregnancy until IPTp intake. Genotypes were compared in each woman, according to band size and number for each of the allelic families of *msp1* and *msp2*. Gel photographs were scored by visual comparison of DNA fragments obtained from baseline and during pregnancy. Unamplified samples and those showing the same *msp2* profile on both consecutive samples were analyzed on the *msp1* block 2 markers. An infection was considered persistent when identical prepregnancy allele(s) were found in pregnancy samples, the 2 patterns being either completely identical or containing some missing or additional genotypes. Infections were considered new if the allelic pattern at both loci differed completely between the prepregnancy and pregnancy samples.

Statistical Analysis

The prevalence of *Plasmodium* infection at different time points was determined according to the number of pregnant women seen at that particular time point. We initially used univariate

analysis to present the baseline characteristics of the study population. Then we analyzed the risk of *P. falciparum* infection during pregnancy based on parasitological status at enrollment, using a logistic mixed regression model. The analysis was adjusted for gestational age and parity status. All statistical analyses were done with Stata software, version 13.0 (StataCorp). All tests were conducted using a .05 level of significance.

RESULTS

Baseline Characteristics of Study Population, Sample Collection, and Follow-up

A total of 275 nulligravidae were included in cohort 1. Within a year, 73 women became pregnant, and 68 were included in the secondary cohort; thus, recruitment to the primary cohort was terminated. The time elapsed between inclusion and occurrence of pregnancy was 3 months on average (median, 66 days; interquartile range, 43–130 days). Of the 68 pregnancies included, 15 withdrawals of consent and 16 spontaneous abortions or obstetric pathologies led to 37 deliveries, including 2 neonatal deaths (Figure 1). The monitoring of the primigravid cohort was more complex than anticipated and resulted in high and unexpected attrition. The characteristics of women admitted to cohorts 1 and 2 are presented in Table 1.

P. falciparum Burden in the Study Population

Sixty-eight nonpregnant women who became pregnant for the first time were included for further follow-up (Table 1). The prevalence rates of *P. falciparum* infection at inclusion in cohort 1 were 15% by microscopy and 66% by real-time PCR targeting the 18S ribosomal RNA gene. At inclusion in cohort 2, the first month of pregnancy, these rates were 12% and 58%, respectively. The prevalence rates of microscopic infections increased throughout the first trimester of pregnancy, reaching 29% before the introduction of IPTp at about the fourth month of pregnancy. The increase in rates of microscopic infections during this period is clearly in opposition to that of submicroscopic infections, which tended rather to decrease. After IPTp administration, all types of infections decreased, but they increased again toward the end of the pregnancy, especially submicroscopic infections (Figure 2).

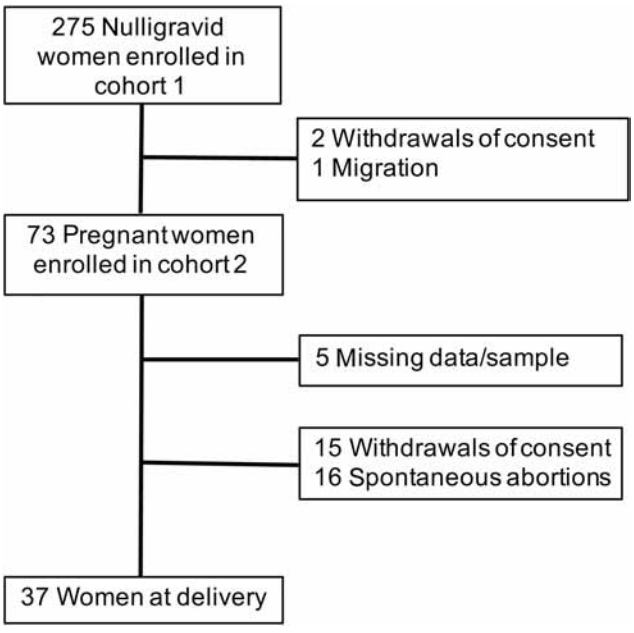

Figure 1. Study profile.

When cohort 2 is stratified into 2 groups according to parasitological status at enrollment, the follow-up patterns show a lower prevalence of microscopic infections in women who were negative at enrollment, compared with those who had parasites before pregnancy (Figure 3). This observation is confirmed by logistic regression analyses that demonstrate an increased risk of infection in women who were infected before pregnancy. Comparable risks were observed when all microscopic infections were recorded during pregnancy (odds ratio, 6.5 [P = .047] for microscopically positive at enrollment and 3.51 [P = .05] for submicroscopic at enrollment) (Table 2). Similar trends were observed when only infections recorded before IPTp initiation were considered.

Genotyping of Recurrent Infections From Women Infected at Baseline

Consecutive samples from 18 women presenting with a microscopic (n = 7) or submicroscopic (n = 11) infection at both enrollment and at least once before IPTp uptake were sequentially genotyped by amplifying the *msp2* and *msp1* (block 2) genes. The mean multiplicity of infection (standard deviation) was 4.3 (1.4) before pregnancy, and 3.1 (1.5) during pregnancy. Overall, persistent infections with ≥1 common allele before and during pregnancy were more frequent than new infections (13 [72%] vs 5 [28%]). Moreover, among the 7 women microscopically infected before pregnancy, infections in all but 1 remained detectable by microscopy, and the mean parasite density increased >20-fold, from 253/μL to 5411/μL. All 11 submicroscopic infections before pregnancy became microscopically detectable during pregnancy, with a mean parasite density of 1228/μL (Table 3).

DISCUSSION

Susceptibility to malaria infections regarding the parasite prevalence and parasite density is increased during pregnancy, representing a major public health problem, with weighty consequences for the mother, the fetus, and the newborn. Understanding the risk factors associated with the occurrence of malaria infections during pregnancy is critical to the development of effective preventive strategies. The WHO recommends that pregnant women use insecticide-treated mosquito nets and IPTp-SP in all areas of Africa with moderate to high malaria transmission. Based on the currently available piece of evidence, the preventive clinical efficacy of IPTp-SP in pregnant women has been demonstrated and is conserved even in areas

Table 1. Baseline Characteristics of Women admitted to Cohorts

Characteristic	Primary Cohort (n = 275)	Secondary Cohort (n = 68)
Age, mean (SD), y	21.2 (0.4)	21.1 (0.9)
Hemoglobin, median (IQR), g/dL	11.9 (11.1–11.9)	11.6 (11.3–12.3)
Educational level, No. (%)		
None	231 (84)	56 (81.2)
Primary school	31 (11.3)	8 (11.6)
Secondary school	13 (4.7)	5 (7.2)
Season of enrollment, No (%)		
Rainy season	59 (21.5)	47 (68.1)
Dry season	216 (75.5)	22 (31.9)
Plasmodium falciparum prevalence, %		
By microscopy	15	12
By real-time PCR	66	58
P. falciparum density, median (IQR), parasites/μL		
By microscopy	249 (88–567)	1070 (13–4242)
By real-time PCR[a]	154 (48–1238)	70 (17–360)

Abbreviations: IQR, interquartile range; PCR, polymerase chain reaction; SD, standard deviation.

[a]For submicroscopic infections, *P. falciparum* densities are 89/μL (33–239/μL) for cohort 1, and 37/μL (13–232/μL) for cohort 2

where *P. falciparum*–resistant genotypes with quintuple *dhps/dhps* mutations are prevalent, but it is vanishing for those with sextuple ones [12], although the parasitological efficacy is compromised [13]. IPTp-SP for pregnant women reduces episodes of maternal malaria, maternal and fetal anemia, placental parasitemia, low birth weight, and neonatal death [14, 15]. However, this strategy is also facing the problem of its application safety window, which excludes the first trimester of pregnancy, when infections are equally harmful to a pregnancy.

In the current study, we enrolled nulligravid women with a desire to procreate, and we followed them up until conception and throughout their first pregnancy. This study thus made it possible to characterize the dynamics of *P. falciparum* infections in this population of first-time pregnant women, those most at risk of malaria infections. The first observation is the demonstrated feasibility of this kind of longitudinal study in Africa, which is among the first such studies. In this study design, a fertility rate of 26.5% (73 of 275 women) within the window of observation was observed, a very satisfactory indicator for future studies to evaluate the effectiveness of preventive strategies deployed during a preconceptional period. Our findings on the dynamics of malaria infections are in line with past notions of increased susceptibility during pregnancy, with a 2-fold increase in the prevalence of microscopic infections between baseline and the first trimester of pregnancy, before IPTp initiation. We also confirmed that submicroscopic infections are more frequent than microscopic infections, especially before pregnancy. During the first trimester of pregnancy the relative prevalence of submicroscopic and microscopic infections evolved differently, so that the overall prevalence of infections remained roughly constant.

Our study clearly shows a previously unknown association between the parasitological status of women before pregnancy and the occurrence of microscopic infections subsequently in a future pregnancy. Furthermore, we show that infections

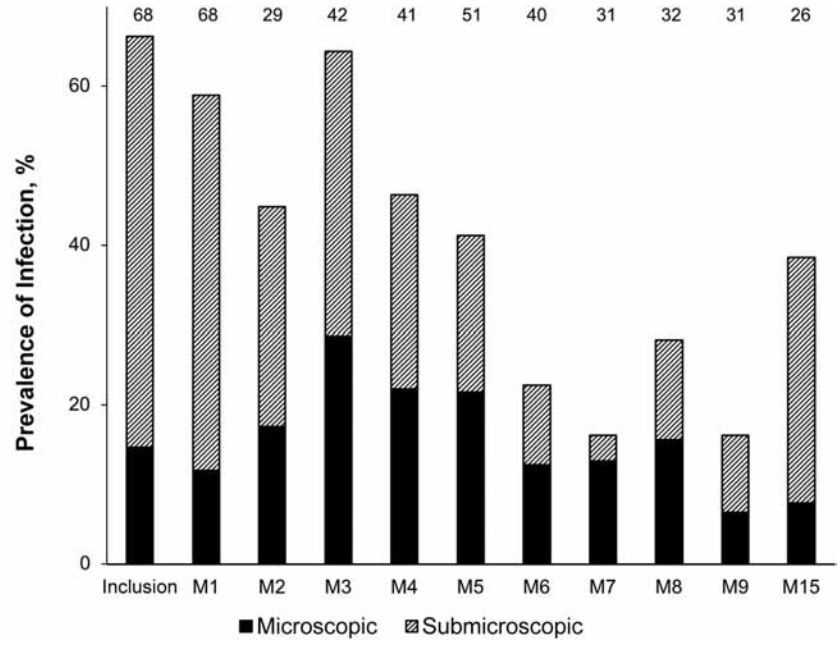

Figure 2. Prevalence rates of *Plasmodium falciparum* infections at inclusion before pregnancy, each month of pregnancy (M1–M9), and 6 months after delivery (M15), determined using microscopy or real-time polymerase chain reaction targeting the 18S ribosomal DNA gene (for submicroscopic infections). The number of women followed up is indicated above each bar.

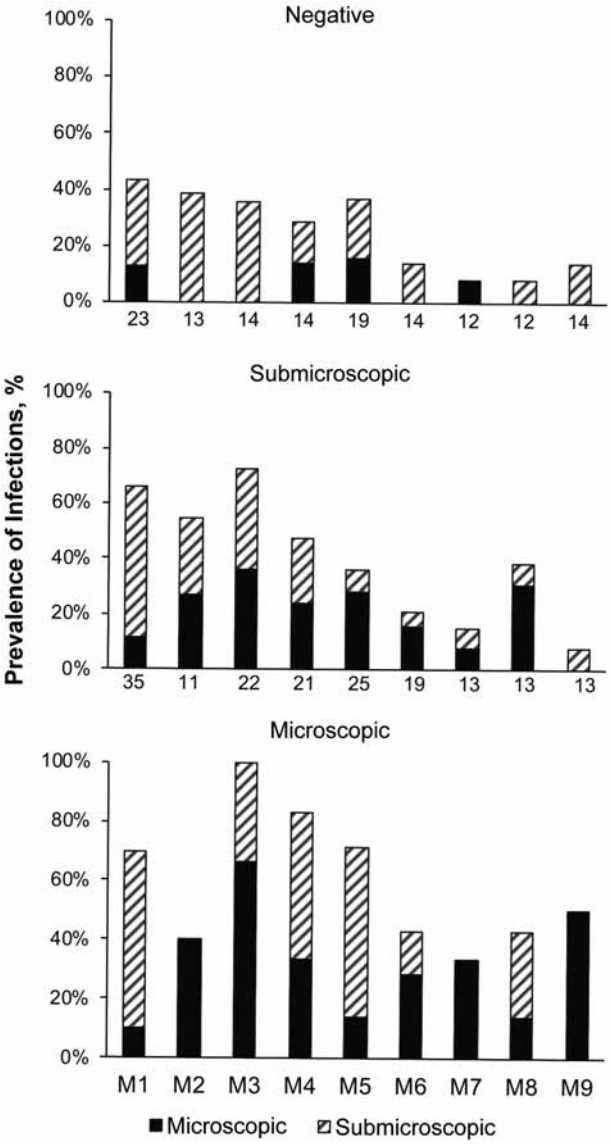

Figure 3. Prevalence rates of *Plasmodium falciparum* infections during pregnancy using microscopy or real-time polymerase chain reaction targeting the 18S ribosomal DNA gene (submicroscopic), by parasitological status at enrollment. The number of women followed up in each subgroup is indicated beneath each bar.

occurring during early pregnancy in areas with high transmissions, such as Benin, are mainly derived from asymptomatic carriage of parasites before conception, including infections at very low densities, commonly known as "submicroscopic." Although it has been suggested that a large percentage of malaria infections carried in early pregnancy might be acquired before pregnancy [16, 17], no formal demonstration has been reported so far. These suggestions were based on seasonal transmission models and did not rule out the total lack of transmission during the dry season. More recent work by our team also suggested that women infected before pregnancy were more likely to be infected during early pregnancy, but it did not rule out the possibility that this was not due mainly to an increased exposure [18].

In our study, parasitological status was determined by microscopy and PCR at the time of inclusion in both cohorts (Table 1). The primary cohort was recruited mainly during the dry season for logistical reasons. Inclusion in the secondary cohort occurred at the time of onset of pregnancy, mostly during the rainy season, when malaria transmission was the highest. Although we could have expected a higher prevalence of microscopic and/or submicroscopic malaria infection among young women included during the rainy season, malaria prevalence was similar in the 2 cohorts before increasing during the first trimester of pregnancy. However, the link established in the present study between these parasite isolates and those present before pregnancy by molecular genotyping definitely comes to demonstrate and establish this fact (Table 3), clearly pointing to asymptomatic carriage before pregnancy as a major factor of infection during early pregnancy, certainly more substantial than transmission variations. Although the WHO is currently recommending the universal use of bed nets to prevent malaria across sub-Saharan Africa, the usage has consistently been lowest in adolescents and young adults [19, 20].

The current study identifies women of childbearing age as a target of choice for deploying specific strategies to reduce infections during pregnancy. Epidemiological evidence of a high prevalence of asymptomatic infections (then untreated) in adolescents and young women, before first pregnancy, further support this observation [19, 21]. Thus, a preventive strategy could involve the use of drugs in the short term or the use of long-term effective vaccines, when available, in the long term. A vaccine earmarked for preventing malaria during pregnancy, to be administered to adolescent girls, would probably be an intervention of choice, but this is not currently available, although it is in the early phases of clinical testing [3, 4]. Alternatively, presumptive administration of a curative antimalarial regimen, probably using an artemisinin-based combination therapy, could be effective in clearing parasitemia in the preconceptional period, and consequently in reducing the burden of malaria parasite infections during the first trimester of pregnancy, before initiation of IPTp. The exact target population and time frame of this treatment administration remain to be determined.

Clonal phenotypic variation in *P. falciparum,* supported by the mutually exclusive expression of genes from multigene families,

Table 2. Risk of *Plasmodium falciparum* Infection During Pregnancy, by Parasitological Status at Enrollment, Determined Using a Logistic Mixed Regression Model

Risk Factor	OR (95% CI)	*P* Value
Type of infection at inclusion		
None	Reference	…
Microscopic	6.5 (1.03–41.35)	.047
Submicroscopic	3.06 (.99–9.52)	.05
Season of infection in pregnancy		
Dry	Reference	…
Rainy	0.55 (.18–1.72)	.31

Abbreviations: CI, confidence interval; OR, odds ratio.

Table 3. Genotyping of Recurrent Infections From Women Infected Before Pregnancy[a]

	Infections Before Pregnancy			Infections During Pregnancy			
Woman	Infection Type	Parasite Density, Parasites/μL[b]	Genotypes, No.	Infection Type	Parasite Density, Parasites/μL[b]	Genotypes, No.	Persistent or Reinfection
A021	Microscopic	249	4	Submicroscopic	0	3	Persistent
A023	Microscopic	40	5	Microscopic	26 280	5	Persistent
A055	Microscopic	662	4	Microscopic	1059	4	Persistent
A071	Microscopic	268	4	Microscopic	177	3	Reinfection
A135	Microscopic	168	2	Microscopic	1625	2	Reinfection
B037	Microscopic	343	5	Microscopic	2235	6	Persistent
B093	Microscopic	45	4	Microscopic	1089	3	Persistent
A069	Submicroscopic	0	6	Microscopic	258	2	Persistent
A075	Submicroscopic	0	2	Microscopic	1656	2	Persistent
A104	Submicroscopic	0	5	Microscopic	160	1	Reinfection
A111	Submicroscopic	0	4	Microscopic	12	3	Persistent
A119	Submicroscopic	0	7	Microscopic	222	6	Persistent
B055	Submicroscopic	0	4	Microscopic	15	3	Reinfection
B067	Submicroscopic	0	5	Microscopic	1493	5	Persistent
B088	Submicroscopic	0	5	Microscopic	4910	5	Persistent
B099	Submicroscopic	0	6	Microscopic	159	3	Persistent
B122	Submicroscopic	0	4	Microscopic	12	2	Persistent
B128	Submicroscopic	0	2	Microscopic	3510	4	Reinfection

[a]The number of genotypes was derived from the analysis of *msp1* and *msp2* allelic families after genotyping (see Methods). Persistent infections were defined as prepregnancy alleles (either all or not) present in pregnancy samples. Reinfections where defined as fully different allelic patterns between the prepregnancy and pregnancy samples.

[b]Parasite density as defined by microscopy.

such as *var* genes, is known to be the mechanism underlying the variable clinical forms of *P. falciparum* malaria [22]. Several studies have showed that parasites infecting pregnant women specifically express the *var2csa* member, in contrast to parasites infecting nonpregnant individuals [23, 24]. Our observations strongly suggest that these pregnancy-specific parasite phenotypes, most probably representing a minor population in the parasite isolates before pregnancy, can be rapidly selected for in vivo when favorable physiological conditions are met. In this case, the appearance of the placenta will allow the exposure of such parasites to chondroitin sulfate A receptors that are specific to this organ. Such phenotype switching is widely reported with in vitro manipulations, with the panning and generation of selected cytoadherence phenotypes from the same parasite clone [25, 26]; no experimental data from in vivo conditions is currently available. Although our current findings do not properly demonstrate this phenotypic switch in vivo, the persistence of preconceptional genotypes and their expansion during pregnancy strongly suggest it.

An additional limitation involves the limited sample size of pregnant women who were followed up. However, the study brings a proof of concept that such a preconceptional cohort is achievable in an African context. Importantly, this allows the study of the very first weeks of pregnancy, a time frame that has hitherto never been investigated. With this sample size, we were still able to demonstrate that malaria infections before pregnancy persisted during pregnancy and were potentially harmful.

To conclude, we found a substantial burden of microscopic and submicroscopic malaria infection in nulligravid women from south Benin. When these women became pregnant, and before first IPTp administration, the overall prevalence of infections remained roughly similar, but the proportion of microscopic infections, with a higher parasite density in blood, clearly increased. Infections occurring during early pregnancy were derived mainly from parasites infecting a woman before conception. Preventive strategies targeting nonpregnant women with a desire for conception need to be designed to allow the best management of this population before contact with antenatal clinics.

Notes

Acknowledgments. This study is part of the PlacMalVac collaborative project, "Clinical Development of a VAR2CSA-Based Placental Malaria Vaccine." We thank Parfait Houngbegnon for assistance in data analysis.

Financial support. This study was supported by the European Seventh Framework Programme (contract 304815).

Potential conflicts of interest. All authors: No reported conflicts of interest. 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

1. Updated WHO policy recommendation. Intermittent preventive treatment of malaria in pregnancy using sulfadoxine-pyrimethamine (IPTp-SP). Available at: http://www.who.int/malaria/publications/atoz/who_iptp_sp_policy_recommendation/en/. Accessed October 2012.
2. Cottrell G, Deloron P, Fievet N, Sow S, Gaye O, Le Hesran JY. Predictive power of *Plasmodium falciparum* infection during early, middle and late pregnancy on placental infection at delivery. Acta Trop **2006**; 98:255–60.
3. Nielsen MA, Resende M, de Jongh WA, et al. The influence of sub-unit composition and expression system on the functional antibody response in the development of a VAR2CSA based *Plasmodium falciparum* placental malaria vaccine. PLoS One **2015**; 10:e0135406.

4. Tuikue-Ndam N, Deloron P. Developing vaccines to prevent malaria in pregnant women. Expert Opin Biol Ther **2015**; 15:1173–82.
5. World Health Organization. Malaria in pregnant women, **2016**. Available at: http://www.who.int/malaria/areas/high_risk_groups/pregnancy/en/. Accessed 25 May 2017.
6. Cottrell G, Moussiliou A, Luty AJF, et al. Submicroscopic malaria infections in pregnant women have serious impact on anemia, prematurity and low birth weight of the offspring: a longitudinal study in Benin. Clin Infect Dis **2015**; 60:1481–8.
7. Rogerson SJ, Desai M, Mayor A, Sicuri E, Taylor SM, van Eijk AM. Burden, pathology, and costs of malaria in pregnancy: new developments for an old problem. Lancet Infect Dis **2018**; 18:e107–18.
8. Djènontin A, Bio-Bangana S, Moiroux N, et al. *Culicidae* diversity, malaria transmission and insecticide resistance alleles in malaria vectors in Ouidah-Kpomasse-Tori district from Benin (West Africa): a pre-intervention study. Parasit Vectors **2010**; 3:83.
9. Diallo A, Ndam NT, Moussiliou A, et al. Asymptomatic carriage of *Plasmodium* in urban Dakar: the risk of malaria should not be underestimated. PLoS One **2012**; 7:e31100.
10. Taylor SM, Mayor A, Mombo-Ngoma G, et al. A quality control program within a clinical trial consortium for PCR protocols to detect *Plasmodium* species. J Clin Microbiol **2014**; 52:2144–9.
11. Snounou G, Zhu X, Siripoon N, et al. Biased distribution of *msp1* and *msp2* allelic variants in *Plasmodium falciparum* populations in Thailand. Trans R Soc Trop Med Hyg **1999**; 93:369–74.
12. Minja DT, Schmiegelow C, Mmbando B, et al. *Plasmodium falciparum* mutant haplotype infection during pregnancy associated with reduced birthweight, Tanzania. Emerg Infect Dis **2013**; 19:1446–54.
13. Moussiliou A, De Tove YS, Doritchamou J, et al. High rates of parasite recrudescence following intermittent preventive treatment with sulphadoxine-pyrimethamine during pregnancy in Benin. Malar J **2013**; 12:195.
14. Schultz LJ, Steketee RW, Macheso A, Kazembe P, Chitsulo L, Wirima JJ. The efficacy of antimalarial regimens containing sulfadoxine-pyrimethamine and/or chloroquine in preventing peripheral and placental Plasmodium falciparum infection among pregnant women in Malawi. Am J Trop Med Hyg **1994**; 51:515–22.
15. Shulman CE, Dorman EK, Cutts F, et al. Intermittent sulphadoxine-pyrimethamine to prevent severe anaemia secondary to malaria in pregnancy: a randomised placebo-controlled trial. Lancet **1999**; 353:632–6.
16. Walker PG, Griffin JT, Cairns M, et al. A model of parity-dependent immunity to placental malaria. Nat Commun **2013**; 4:1609.
17. Berry I, Walker P, Tagbor H, et al. Seasonal dynamics of malaria in pregnancy in West Africa: evidence for carriage of infections acquired before pregnancy until first contact with antenatal care. Am J Trop Med Hyg **2018**; 98:534–42.
18. Accrombessi M, Fievet N, Yovo E, et al. Prevalence and associated risk factors of malaria in the first trimester of pregnancy: a preconceptional cohort study in Benin. J Infect Dis **2018**; 217:1309–17.
19. Nankabirwa J, Brooker SJ, Clarke SE, et al. Malaria in school-age children in Africa: an increasingly important challenge. Trop Med Int Health **2014**; 19:1294–309.
20. Tchinda VH, Socpa A, Keundo AA, et al. Factors associated to bed net use in Cameroon: a retrospective study in Mfou health district in the Centre Region. Pan Afr Med J **2012**; 12:112.
21. Pullan RL, Bukirwa H, Staedke SG, Snow RW, Brooker S. *Plasmodium* infection and its risk factors in eastern Uganda. Malar J **2010**; 9:2.
22. Chen Q, Schlichtherle M, Wahlgren M. Molecular aspects of severe malaria. Clin Microbiol Rev **2000**; 13:439–50.
23. Salanti A, Dahlbäck M, Turner L, et al. Evidence for the involvement of VAR2CSA in pregnancy-associated malaria. J Exp Med **2004**; 200:1197–203.
24. Tuikue Ndam NG, Salanti A, Bertin G, et al. High level of *var2csa* transcription by *Plasmodium falciparum* isolated from the placenta. J Infect Dis **2005**; 192:331–5.
25. Janes JH, Wang CP, Levin-Edens E, et al. Investigating the host binding signature on the *Plasmodium falciparum* PfEMP1 protein family. PLoS Pathog **2011**; 7:e1002032.
26. Gölnitz U, Albrecht L, Wunderlich G. *Var* transcription profiling of *Plasmodium falciparum* 3D7: assignment of cytoadherent phenotypes to dominant transcripts. Malar J **2008**; 7:14.