Longitudinal Studies of *Plasmodium falciparum* Malaria in Pregnant Women Living in a Rural Cameroonian Village with High Perennial Transmission

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Abstract. A prospective longitudinal study of *Plasmodium falciparum* in pregnant women was conducted in the rural village of Ngali II, where malaria is hyperendemic and individuals receive ~0.7 infectious mosquito bites/person/day throughout the year. Pregnant women ($N = 60$; 19 primigravidae, 41 multigravidae) were enrolled early in pregnancy (median 14 wk) and were followed monthly, with 38 women followed through term (5.7 ± 1.1 prenatal visits and delivery). The total number of times primigravidae were slide-positive during pregnancy was higher than multigravidae (3.3 ± 1.1 versus 1.3 ± 1.3 times; $P < 0.001$), but no difference in the number of polymerase chain reaction-positive cases (4.6 ± 1.7 and 3.4 ± 1.7 times, $P = 0.106$) or total genotypes they harbored (8.9 ± 3.2 and 7.0 ± 2.9) was found. Only 7.9% women developed symptomatic infections. All primigravidae and 38% multigravidae were placental malaria-positive at delivery ($P = 0.009$). Genotyping showed that 77% of placental parasites were acquired ≥ 30 wks in pregnancy. These results help identify the extent of malaria-associated changes women experience during pregnancy.

INTRODUCTION

In sub-Saharan Africa, pregnant women and the developing fetus suffer from the adverse effects of *Plasmodium falciparum* infections.^{1–3} Numerous cross-sectional and case-control studies have documented that pregnant women are more susceptible to malaria than non-pregnant women, and that young primigravidae are more likely to be slide-positive, have higher parasitemias, and develop anemia than multigravidae (reviewed in References 4–6). Epidemiological studies also show that the accumulation of *P. falciparum*-infected erythrocytes in the intervillous space (IVS) of the placenta, especially in paucigravidae, increases a woman's risk of premature deliveries and low birth weight (LBW) babies.^{7–9} Thus, substantial information has been obtained about malaria in pregnant women under different environmental conditions. Surprisingly, however, few comprehensive studies have followed individual women throughout the course of pregnancy. Perhaps the best comprehensive study was conducted 40 years ago by Gilles and colleagues¹⁰ in Nigeria where they monitored hematological changes, anemia, erythrocyte survival rate, antimalarial antibodies, and pregnancy outcome in cohorts of primigravidae. Similar information for multigravidae has not been reported. Therefore, information about the malaria experience of individual women remains limited. For example, how often does a pregnant woman become infected? How many different strains (genotypes) of *P. falciparum* does she harbor during the course of pregnancy? Are parasite genotypes rapidly cleared or do they persist until delivery? How frequently does a woman develop clinical symptoms of malaria and anemia; and how does this relate to placental pathology and pregnancy outcome? Accordingly, we undertook a comprehensive prospective longitudinal study in pregnant women residing in the rural village of Ngali II in Cameroon. Data describing the influence of malaria on pregnant women who were

followed from the first trimester through delivery are presented; including prevalence of slide and submicroscopic infections, number of circulating parasite genotypes, clinical symptoms, presence of anemia, delivery outcomes, and placental pathology. Results on cellular and humoral immune responses of these women will be reported subsequently. Because this is the first study in Ngali II, data on transmission dynamics and prevalence of *P. falciparum* in the general population are also provided. Results from this study help answer some of the previous questions about *P. falciparum* infections in individual women. They also provide baseline information about the burden of malaria in pregnant women living in a typical rural African setting before the adaptation of intermittent preventive therapy and insecticide-treated bed nets.

MATERIAL AND METHODS

Study population. Between January 2001 and May 2005, longitudinal studies were conducted in Ngali II, a rural agricultural village located 03°18'N, 11°35'E 30 km north-east of Yaoundé in the Soa health district. This medium-sized village (~200 Km²) has ~3,500 inhabitants of the Ewondo clan who live in small wood-mud houses, most of which lack water and electricity. The village is bordered by the Mefou Afamba River from which several small streams irrigate parts of the village. Cassava and its by-products provide a staple diet. The climate is equatorial with annual temperatures ranging from 18 to 30°C. Rainfall averages 1,670 mm² annually, but the length of the two wet and two dry seasons differs annually. The village is divided into four blocks. In this study, rainfall was recorded daily within each of the four blocks using rain gauges (recorded in mm) and averaged monthly during the 4-year study period. In Ngali II, there is one government health center that provides outpatient consultations and a small pharmacy with a limited supply of drugs. During the project, the Cameroonian medical team participating in this study offered free consultation to the community at least once a week and some treatments, including antimalarial drugs and iron supplements, were provided. Individuals participating

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in the study gave written informed consent. The study was approved by the National Ethics Committee of Cameroon and the Institutional Review Board (IRB), Georgetown University.

Entomological studies. Entomological surveys were conducted in Ngali II from May to August 2004 and from October 2004 to February 2005. Mosquitoes were collected indoors from 6:00 PM to 6:00 AM on five consecutive nights each month with eight collectors per night using the human landing-catch method. Following collection, mosquitoes were identified as anophelines, *Culex* sp. or *Mansonia* sp. The anophelines were further divided morphologically into groups and members of the *Anopheles gambiae* complex were identified to species level using the polymerase chain reaction (PCR).¹¹ All mosquitoes from the *An. gambiae* s.s. group were further identified by molecular form (M or S).¹² The presence of *Plasmodium* sporozoites was assessed by enzyme-linked immunosorbent assay (ELISA) using the head and thorax of each mosquito.¹³ The cut-off for positivity was the mean of five negative control mosquitoes plus three standard deviations (SD).

Cross-sectional survey of the population. The prevalence of malaria within the village was determined by conducting four door-to-door household surveys in August–September 1998 (before initiating the study), in March–April 2000, and in August–September 2002 and 2004. During the surveys, household demographics were recorded and finger-prick blood was collected in heparinized capillary tubes from all individuals who consented to participate. Thick and thin blood smears were prepared, stained with Diff-quick (Baxter Scientific, McGaw Park, IL), and examined for malarial parasites. If parasites were detected, the number of parasites per 200 leukocytes was determined and converted to number of parasites/ μ L assuming a mean leukocyte count of 6,000/ mm^3 . Capillary tubes were centrifuged, hematocrit determined, and prevalence of anemia (i.e., individuals with <30% hematocrit) was recorded. Informed consent was obtained from adults and parents or legal guardians of minors.

Design of longitudinal studies in pregnant women. The study was conducted over a 40-month period at the government-supported clinic in Ngali II, where a monthly antenatal clinic is held. Each month, the project was explained to women attending the clinic who were in their first trimester of pregnancy. An average of three women enrolled each month. The women were followed monthly at the antenatal clinic and at delivery. If a woman became ill between visits, she contacted the local health care worker who made arrangements for her to be seen by a physician. Thus, women were continuously enrolled and followed monthly, and when they were ill, throughout the wet and dry seasons over a 3.3-year period. Upon enrollment, the women were asked to complete a questionnaire about their previous malaria and pregnancy histories. The women were also informed about the potential severe consequences of malaria during pregnancy and methods for preventing malaria were discussed. At each monthly antenatal visit thereafter, women were questioned about their pregnancy, including symptoms associated with malaria; use of antimalarial drugs, bed nets, and other preventative measures; and various sociological questions. Data from physical examinations were recorded, e.g., blood pressure, hemoglobin level (HemoCue, Lake Forest, CA), height, weight gain, and body mass index (BMI). A blood sample was taken by veni-puncture on odd

numbered months and finger-stick on even number visits. Laboratory tests included hematocrit, total white blood cell (WBC) count, ABO blood group, and sickle cell trait (A/A versus A/S by strip electrophoresis (Helena, Beaumont, TX)). To detect *P. falciparum* infections, thick and thin blood smears were prepared, stained with Dif-Quick, and examined for parasites by two microscopists. If parasites were present, the number of parasites per 200 WBC was determined and the number of parasites/ μ L of blood was calculated using each woman's WBC count. If positive, the results were reported to the attending health care professional who prescribed antimalarial treatment according to the Ministry of Health policy. Prior to 2002, chloroquine (CQ) was the first-line drug for uncomplicated malaria. However, between 1999 and 2004 CQ resistance reached 48.6% in children <10 years of age and between 2000 and 2004 64% of parasite isolates tested *in vitro* were CQ resistant.^{14,15} Because of increasing drug resistance, amodiaquine (AQ) became the official drug of choice (2002–2004). Since 2004, artemisinin plus AQ is the recommended first-line drug.^{14,15} Women contacted the village health worker if they became ill to receive treatment of malaria or other conditions.

At delivery, the weight of the newborn was recorded and a biopsy of the placenta was collected. A portion of the placenta was used to prepare impression smears that were stained with Dif-quick, examined for parasites, and the percent parasitemia was determined. A portion of the placental biopsy was fixed in buffered formalin, sectioned, stained with hematoxylin/eosin and Giemsa, and examined for parasites and pathology. Each placenta was scored as having either an active (parasites, minimal hemozoin pigment, normal number macrophages), chronic (parasites, pigment in macrophages), past infections (no parasites, pigment in macrophages), or having no evidence of infection (no parasites, no pigment) as previously described by Bulmer and others¹⁶ and Ismail and others.¹⁷ Approximately 45% of births occurred outside of the Ngali II Health Center, making it impossible to collect placental biopsies. When this happened, the family was visited the next day to obtain information on the delivery and newborn. Information on sample size is provided in the text, figure legends, and tables.

Detection and genotyping of parasites by PCR. Blood samples collected during the course of pregnancy and at delivery were examined by PCR for *P. falciparum*. In brief, DNA was extracted from 50 to 200 μ L of frozen erythrocytes using Puregene DNA isolation kits (Gentra Systems Corp., Minneapolis, MN). When >200 μ L of red blood cell (RBC) were available, DNA from some samples was isolated using the BioRobot EZI workstation (Qiagen, Valencia, CA). To detect parasites, a nested PCR reaction that amplified the *P. falciparum* small subunit ribosomal RNA gene was used.^{18,19} Women were considered to have submicroscopic infections if they were PCR-positive but blood smear negative. If parasites were detected, the number of different parasite strains/genotypes present, i.e., the multiplicity of infectivity (MoI) was determined on the basis of polymorphisms in *msp1* (Block 2 and 4: K1, MAD20, and RO33) and *msp2* (Fc27 and 3D7).^{19,20} This assay is a standard approach for studying genetic diversity of *P. falciparum*,^{18,20} including in pregnant women.^{19,21–25} The number of alleles at the two loci was determined and the locus with the largest number of bands was used to estimate the MoI. In addition, the C-terminus of MSP-1 was sequenced and the MSP1-19 type was determined based on amino acids at

position 294, 341, 349, 350, 351, and 366 of the MSP-1_{42-3D7} reference sequence.^{26,27}

Statistical analysis. Pearson's χ^2 test was used for between-group comparisons for categorical parameters (e.g., malaria prevalence, anemia, LBW, premature deliveries, etc). Wilcoxon rank-sum test was used for between-group comparisons of continuous parameters, (e.g., birth weight, parasitemia, length of pregnancy, etc). To account for a lack of independence among repeat visits for a subject, the type 3 likelihood ratio test (LRT) based on unstructured generalized estimation equation was used to compare percent slide-positive peripheral malaria, peripheral malaria parasitemia, and number of peripheral malaria genotypes between primigravidae and multigravidae throughout pregnancy. A Poisson multivariate regression model was used to evaluate the association of higher rates of malaria slide positivity during pregnancy in primigravidae by including age, concerned with malaria infection, and use of chemoprophylaxis during the pregnancy. All descriptive and inferential statistics were performed using SAS 9.2 (SAS Institute, Cary, NC).

RESULTS

Entomological vectors and inoculation rates. Rainfall measurements identified two annual wet (April–June and September–November) and two drier (December–March and July–August) seasons (Figure 1A). The annual pattern was relatively consistent, but the second wet season occurred earlier in some years than others. Entomological studies conducted in

2004 resulted in the capture of 775 mosquitoes. *Anopheles nili* was the most abundant species that transmits malaria (45.7%), followed by *Anopheles funestus* (16%), *An. gambiae* (14.2%), and *Anopheles moucheti* (1.9%) (Figure 1B). All *An. gambiae* collected were identified as *An. gambiae* s.s. and of the M molecular form. The distribution of the malaria vectors varied throughout the year, but the change in numbers did not follow the seasons. The average monthly entomological inoculation rate (EIR) was 21.3 infectious bites/person/mon (ib/p/mon), with rates lower in the dry seasons (e.g., July [9.4 ib/p/mon] and August [14 ib/p/mon]) compared with the other months) ($P = 0.046$). The annual number of infective bites/person/year (ib/p/yr) for *An. nili*, *An. funestus*, *An. gambiae*, and *An. moucheti* were 144 ib/p/y, 70 ib/p/y, 38 ib/p/y, and 5 ib/p/y, respectively. Overall, results showed those individuals in Ngali II received ~257 *P. falciparum* infectious bites/p/yr or 0.7 ib/p/day throughout the year.

Prevalence of malaria in Ngali II. The prevalence of slide-positive *P. falciparum* infections was determined at the end of the dry season in 1998, 2002, and 2004 and during the rainy season 2000 (Figure 2). Among the four surveys, *P. falciparum* was detected in an average ($\pm$ SEM) of $75 \pm 6\%$ of children 1–4 years of age (range 62–83%) and $68 \pm 4\%$ of those 5–14 years of age (range 52–77%). In young adults (15–19 yr), the prevalence declined to $43 \pm 2\%$ (range 40–45%) and was $26 \pm 2\%$ (range 8–38%) in adult ≥ 30 years of age. Because there was a gradual decline in prevalence between 15 and 39 years of age, adults in this age group, which includes women of child-bearing age, were still acquiring immunity to *P. falciparum* (Figure 2). Minimal variation was found in prevalence of *P. falciparum* at the end of the dry season and rainy season, even though there was a difference in vector transmission rates (Figure 1B). The overall annual prevalence did not differ significantly during the 6-year period of study.

The prevalence of anemia was also recorded. Hematocrits $< 30\%$ were found in $14.4 \pm 3.5\%$ for children between 1 and 4 years of age, but anemia was uncommon in older children 5–14 years of age ($1.0 \pm 0.18\%$) even though more than half of them had asymptomatic infections (Figure 2). Anemia was detected in few adult males ($0.8 \pm 1.3\%$) and females ($3.5 \pm 1.7\%$). Overall, these results showed that *P. falciparum* is hyperendemic (i.e., prevalence $\geq 75\%$ in children 1–4 years of age and perennially transmitted)²⁸ in Ngali II and that residents quickly gained immunity to disease and malaria-associated anemia after repeated malarial exposures.

Characteristics of the pregnant women enrolled in the study.

A total of 60 women were recruited early in pregnancy (median enrollment 14 wk [13.0–16.5 wk]), including 19 primigravidae and 41 multigravidae (mean 3.1 ± 1.7 prior pregnancies, range 1–8). These women had lived in Ngali II for an average of 9.6 yr. They ranged in age from 14 to 38 yr, with 41.7% being lower than 20 yr. The mean age of primigravidae and multigravidae were 18.4 ± 3.5 yr and 25.8 ± 5.4 yr, respectively. The women had an average of 6.5 yr of formal education (range 3–14 yr) and 84.5% listed farming as their primary occupation, 8.6% worked at home, and 6.9% had other employment. None of the women smoked and only 16.7% drank alcohol during pregnancy. Overall, 20.3% of the women had blood type A, 39.0% type B, 0% AB, and 40.7% type O, and 19.3% were carriers of the sickle cell trait (A/S).

Sixty-three percent (38/60) of the women were followed throughout pregnancy with a mean of 5.7 ± 1.1 prenatal visits

FIGURE 1. Rainfall and entomological inoculation rates in Ngali II. (A) Average rainfall (linear mm) showed annual variations between the two wet (shaded areas) and two dry periods. (B) Entomological studies conducted between May 2004 and February 2005 identified four predominant mosquito vectors of *Plasmodium falciparum* including *Anopheles nili*, *Anopheles funestus*, *Anopheles gambiae*, and *Anopheles moucheti*. Estimated entomological inoculation rates (EIR) are shown.

FIGURE 2. Prevalence of malaria in Ngali II. Door-to-door surveys were conducted at the end of the dry season August–September 1998 ($N = 830$ participants), 2002 ($N = 511$), and 2004 ($N = 558$ individuals) and in the wet season March–April 2000 ($N = 774$ individuals). The square highlights the prevalence of malaria in 15–40 year olds, which includes women of childbearing age.

per woman. Data from these women (12 primigravidae and 26 multigravidae) were used in longitudinal analyses. The other 22 women dropped out with a mean of 2.1 ± 0.9 prenatal visits, mostly because they moved from the village. Data from all women were used in cross-sectional analyses.

Use of malaria prevention. A survey was conducted at enrollment and a questionnaire was completed at each prenatal visit to document use of antimalarial preventive measures. At enrollment, 48% (28/59) of the women expressed concerns about having malaria. None of the women used bed nets during pregnancy, primarily because of lack of availability within the village. However, 98% (48/49) reported taking chemoprophylaxis throughout pregnancy. Both primigravid and multigravid women began taking antimalarial drugs at 18.3 ± 4.0 and 18.2 ± 4.1 wk, and continued using them until near term (33.8 ± 5.4 and 35.6 ± 5.8 wk, respectively), with 60% taking CQ, 27% using a combination of CQ and other drugs, and 13% taking other drugs. The use of prophylaxis did not differ between primigravidae and multigravidae.

Prevalence of slide positivity for malaria in women during pregnancy. Among the 60 women enrolled, the prevalence of slide positivity was higher each month in primigravidae ($54\% \pm 7\%$, mean $\pm$ SEM) than in multigravidae ($26 \pm 3\%$) throughout the course of pregnancy ($P < 0.0001$, type 3 LRT) (Figure 3A). Prevalence was also higher in primigravidae than in age-matched adults in the general population ($44 \pm 5\%$; data from four surveys, $N = 174$ women aged 15–22 yr) even though they took antimalarial drugs for prophylaxis and treatment. In contrast, the prevalence of malaria in multigravidae was lower than in age-matched non-pregnant women in the population ($33\% \pm 9\%$; data from four surveys, $N = 193$ women aged 20–32 yr), most likely caused by increased antimalarial usage. Peripheral parasitemias were also significantly higher throughout pregnancy in slide-positive primigravidae ($6,620 \pm 1,220$ parasites/ μ L) than multigravidae ($1,210 \pm 420$ p/ μ L) ($P < 0.0001$, type 3 LRT) (Figure 3B).

In the cohort followed longitudinally, primigravidae became slide-positive for *P. falciparum* early in pregnancy with parasites detected in 53% by the third month (14–17 wk) and all primigravidae by the fifth month (Figure 4A). In contrast,

a gradual increase in the accumulation of slide positivity was found among multigravidae, with only 79% becoming slide-positive before delivery. Primigravidae and multigravidae were blood smear positive 3.3 ± 1.1 and 1.3 ± 1.3 times, respectively during pregnancy ($P < 0.001$), i.e., they were slide-positive at $60\% \pm 18\%$ and $21\% \pm 23\%$ of their prenatal visits. The slide-positive rate in primigravidae remained significantly higher than in multigravidae ($P = 0.046$) after adjusting for age, expressed concern about having malaria, and use of chemoprophylaxis in a multivariate analysis. Slide positivity rates were similar between women followed longitudinally and those in the larger group used in the cross-sectional analysis (Figure 3A). The finding that 92% of primigravidae were slide-positive three or more times compared with only 18.5% of multigravidae, illustrates the extent to which primigravidae were more susceptible to *P. falciparum* than multigravidae, even when CQ and other antimalarial drugs were used.

Prevalence of PCR-detected and submicroscopic infections.

All primigravidae and 97% of multigravidae became PCR-positive for *P. falciparum* before the 26th week of gestation (Figure 4B). Longitudinal analysis estimated that primigravidae and multigravidae were PCR-positive an average of 4.6 ± 1.7 and 3.4 ± 1.7 times (i.e., at 92% and 62% of prenatal visits) before delivery, respectively, or 5.1 ± 1.8 (92%) and 4.1 ± 1.9 times (66%) including delivery. The difference was not statistically significant ($P = 0.106$).

Using microscopy and PCR, *P. falciparum* were detected in the blood of $74\% \pm 3\%$ of pregnant women each month, with an average of $40\% \pm 3\%$ being slide-positive and $34 \pm 4\%$ having submicroscopic infections (Figure 4C). The ratio of microscopic to submicroscopic infections remained relatively consistent throughout pregnancy. For comparison, 36.1% of age-matched non-pregnant women ($N = 16$ women, 40 blood samples) had submicroscopic infections.

Multiplicity of infectivity (MoI) during pregnancy. The MoI were determined using 185 PCR-positive blood samples (Figure 5A). Results showed that MoI in samples collected < 14 wks averaged 4.8 ± 1.3 and 4.0 ± 1.6 parasite genotypes in primigravidae and multigravidae, respectively. Thereafter, the number of genotypes remained relatively constant in the

A Slide-positive for *P. falciparum* **B** Parasitemia

FIGURE 3. Prevalence of slide-positive malaria during the course of pregnancy. (A) Prevalence of slide-positive malaria in primigravidae ($N = 9-16$ women/time point) and multigravidae (19–33 women/time point). DEL = peripheral blood at delivery. The hatched rectangles show the mean prevalence ± 1 SEM for non-pregnant women who are the same age as primigravidae (15–22 yr) and multigravidae (20–32 yr) in the general population (based on an average of the four prevalence studies shown in Figure 2). (B) Mean number of parasites/ μ L for slide-positive women on the basis of data from the 16 primigravidae and 24 multigravidae who became slide-positive.

peripheral blood of primigravidae (mean 3.7 ± 0.2 parasite genotypes) and multigravidae (2.7 ± 0.12 genotypes), with significantly higher numbers found in primigravidae ($P = 0.0002$, type 3 LTR).

Total number of parasite genotypes detected during pregnancy. The total number of different *msp1* and *msp2* genotypes detected in the peripheral blood of each woman during pregnancy was determined (Table 1). It was assumed that if a genotype was detected early in pregnancy and again later, it resulted from the same infection. The total number of parasite genotypes detected by *msp1* and *msp2* alleles was similar (Table 1). Overall, the total number of different parasite genotypes infecting primigravidae and multigravidae during pregnancy was similar, 8.9 ± 3.2 and 7.0 ± 2.9 , respectively.

The number of MSP1-19 variants present in the peripheral blood of 20 pregnant women was determined by DNA sequencing of 75 samples (Table 1). Nine variants were identified, with 43% being EKSNGI (FUP type), 35% QKSNGI (FVO type), and only 2.7% ETSSRL (3D7 type). The remaining six variants were detected in less than 4% of the samples. A difference in the distribution of the variants was found between primigravidae and multigravidae. Both the FUP and FVO types were detected in the blood of all primigravidae

A Accumulative slide-positive **B** Accumulative PCR-positive **C** Slide and submicroscopic infections

FIGURE 4. Longitudinal analysis of slide positivity, polymerase chain reaction (PCR) positivity, and submicroscopic infections during pregnancy: (A) Accumulative time until first infection detected by microscopy ($N = 12$ primigravidae; $N = 26$ multigravidae). (B) Accumulative time until first infection detected by PCR ($N = 12$ primigravidae; $N = 26$ multigravidae). (C) Prevalence of submicroscopic infections. Overall results show that 65–86% of the women were infected with *Plasmodium falciparum* each month of pregnancy (mean of 33 women per time point).

and these were the only variants found. On the other hand, this combination of variants was detected in only 18% of multigravidae, whereas 10 of the 11 multigravidae were infected with one or more of the rare variants. Although parasites were sequenced from only 20 women during the course of

A Mol **B Persistence of parasites**

FIGURE 5. Multiplicity of infectivity (Mol). (A) The mean number of parasite genotypes detected in the peripheral blood of primigravidae and multigravidae at each time point. Mean $\pm$ SD, average of 8 and 17 data points per bar for primigravidae and multigravidae, respectively. (B) At delivery, the number of different parasite genotypes in blood collected from the intervillous space (IVS) was determined. Data were then examined to determine how long each parasite-genotype had persisted in the woman's peripheral blood. A total of 31 parasites could be traced backward through pregnancy by *msp1* and 38 parasites by *msp2* genotyping. Arrows represent the percentage of parasites present at the specified time point that persisted and were detected in the IVS at delivery (Del). Results show that, based on *msp1* and *msp2* genotyping, 19.4% and 13.2%, respectively, of the parasites had persisted from < 22 wk until term. On the other hand, 61.3% and 63.2% of the parasites were detected only at delivery based on *msp1* and *msp2* genotyping of multiple samples from the same woman.

pregnancy, the distribution of MSP1-19 genotypes was clearly different between the two groups.

An attempt was made to determine how long women had been infected with parasite genotypes present in the IVS at term. This was possible because most *msp1* and *msp2* genotypes were detected in < 15% of the women. Because most genotypes were rare in the population, it is likely their presence in the same woman over time was because of persistence and not to new infections. A total of 31 parasites present in blood collected from the IVS could be "traced backward" through pregnancy by *msp1* and 38 parasites by *msp2* genotyping (Figure 5B). Overall, 62% of the parasites in the IVS

had not been detected during pregnancy, but 13.2–19.4% had persisted since the fifth month of pregnancy (Figure 5B).

Clinical manifestations of *P. falciparum* infections. Because symptoms associated with malaria are nonspecific, it is difficult to assess how often they occur in women with mild infections. To investigate the frequency of clinical symptoms, each woman was asked at her monthly prenatal visits if she had had any of the following symptoms within the last 2 days and last 2 wk: fever, chills, headache, vomiting, nausea, diarrhea, lethargy, cough, abdominal pain, myalgia, or puritis. On the basis of 299 responses, 39.2% of the women who were slide-positive and 40.6% who were slide-negative reported having one or more of the symptoms. Only three of the 38 (7.9%) women followed longitudinally were diagnosed as having one or more episodes of malaria, i.e., they were slide-positive, had fever, plus one or more of the above symptoms, for an overall prevalence rate of 2% (5 episodes/299 visits). Thus, there was little evidence that women developed malaria-associated symptoms when they were slide-positive, or that slide-positive and submicroscopic infections ultimately developed into clinical episodes.

With the exception of anemia and hypertension, the clinical parameters studied remained within normal ranges (Table 2). Among the cohort followed longitudinally, 50% of primigravidae were anemic during the second and third trimesters compared with only 16.2% and 7.7% of multigravidae ($P < 0.01$). Five of the 38 women showed signs of hypertension in the second and third trimester, including three primigravidae and two secundigravidae. Fever, severe anemia, and high or low WBC counts were uncommon.

At each visit, maternal weight was recorded and BMI determined. Between the 14th and 38th wk, multigravidae gained more weight (6.5 ± 3.4 kg) than primigravidae (1.9 ± 2.5 kg) ($P = 0.009$ *t* test). The BMI rose from an average of 21.5 to 23.0 in primigravidae and 20.9 to 22.9 in multigravidae ($P = 0.008$ *t* test) during this period. The effect of malaria on weight gain has received little attention. Unfortunately, the effect could not be assessed in primigravidae because they were blood smear positive on multiple occasions, however, multigravidae who were slide-positive only 0–1 times during pregnancy gained 7 ± 6 kg compared with those who were slide-positive ≥ 2 times who gained 5 ± 3 kg. Although the difference was not significant because of wide variation and small sample size, results suggest that malaria may have an impact on maternal weight gain.

Pregnancy outcomes. At delivery, 57% of primigravidae were peripheral blood smear positive, 83% were peripheral blood PCR-positive, and 100% had placental malaria (PM) with evidence of active (50%) and chronic (50%) infections (Table 3). In comparison, only 17% of multigravidae were peripheral blood smear positive, 68% were PCR-positive, and 38% had PM with 19% having active and 19% having chronic infections. Overall, 52% of the placentas of multigravidae showed no evidence of past infections even though 97% of the multigravidae were PCR-positive at least once during

TABLE 1
Total number of parasite genotypes detected in the peripheral blood of women during pregnancy

	MSP1 Mol	MSP2 Mol	MSP1-19 variants	Total
Primigravidae (N = 12)	7.3 $\pm$ 4.1* (range 2–14)	7.3 $\pm$ 2.2 (range 4–11)	2.0 $\pm$ 0 (range 2)	8.9 $\pm$ 3.2 (range 4–14)
Multigravidae (N = 24)	4.8 $\pm$ 3.6 (range 1–13)	6.5 $\pm$ 2.8 (range 1–12)	2.2 $\pm$ 1.0 (range 1–4)	7.0 $\pm$ 2.9 (range 2–13)

* Mean $\pm$ SD based on prenatal visits only (does not include delivery).

TABLE 2

Percentage (%) of women who had the following conditions*

	Second trimester		Third trimester	
	Primi-	Multi-	Primi-	Multi-
Fever: > 37.5°C the result of any cause	22.2	27.0	8.3	3.8
Hypertension: SBP > 140 or DBP > 90†*	16.7	5.4	16.7	3.8
Anemia: Hb < 11g/dL and/or PCV < 30%	50.0‡	16.2‡	50.0‡	7.7‡
Severe anemia: < 7.0 g/dL and/or PCV < 21%	0	0	0	0
Elevated WBC: > 12,500 cells/mm ²	0	2.7	0	7.7
Low WBC: < 4,000 WBC/mm ²	0	0	0	0

* PCV = packed cell volume; WBC = white blood cell.

† SBP = systolic blood pressure; DBP = diastolic blood pressure.

‡ $P < 0.01$, χ^2 test, between primigravidae and multigravidae.

pregnancy. Babies born to primigravidae had a lower birth weight than those born to multigravidae ($P = 0.05$). The impact of PM on birthweight could not be assessed, because all primigravidae were infected at the time of delivery. Most infants were born full term and all survived.

DISCUSSION

This is the first prospective, comprehensive, longitudinal study to use molecular approaches to evaluate the impact of *P. falciparum* malaria in pregnant women exposed to high perennial transmission who received monthly prenatal care. The study was conducted in Ngali II. Entomological and four cross-sectional population-based surveys showed that malar-iometric parameters in this village were similar to those in other African communities with high perennial transmission. Although there are two dry seasons, transmission remained high enough throughout the year for individuals to be bitten by at least one infected mosquito every other night (Figure 2). As a result, residents were continuously being infected with genetically different parasites. The prevalence of slide-positive

malaria decreased until around 25 years of age, indicating that maximal anti-parasite immunity had been obtained by this age. Previous studies have shown that humoral immunity to malarial antigens is acquired until ~30 years of age and is responsible, in part, for anti-parasite immunity.²⁹⁻³¹ The cohort of pregnant women enrolled in this study ranged in age from 14 to 38 yr. Thus, before pregnancy young women were still in the process of “fine-tuning” their antimalarial immune responses, whereas older women had already acquired maximal anti-parasite immunity.

Compared with women in most previous studies, pregnant women in this study had access to good prenatal care, were well informed about the risks of *P. falciparum* infections during pregnancy, and aware of the benefits of malaria preventive measures. All of the women reported purchasing antimalarial drugs for prophylaxis, with most women taking CQ. At monthly prenatal visits, information about the woman's weight, blood pressure, temperature, hemoglobin level, and malaria status by blood smear was obtained, and the attending health care professional discussed the results with the women and prescribed treatment of anemia and malaria as needed. Therefore, results from this study are for a cohort of women who received a very high level of prenatal care. As a result, the prevalence of anemia and LBW babies was lower in this study than reported in other Cameroonian studies where women received lesser prenatal care.^{32,33}

When evaluating the results, an overwhelming difference in the impact of *P. falciparum* malaria on young primigravidae women was seen. Numerous studies have reported that slide positivity is about two times higher in primigravid than multigravid women and that they are more susceptible to anemia (reviewed in References 1-6). Nevertheless, one might have assumed that good prenatal care would help equalize the difference. However, malaria remained strikingly more severe in primigravid women. They were more likely to become infected earlier in pregnancy (Figure 2A and B), be slide-positive more often during pregnancy (Figure 3A), have higher parasitemias (Figure 3B), carry more circulating parasites genotypes (Figure 5A), develop anemia during the second and third trimester (Table 2), and gained less weight ($P = 0.005$). At delivery, all primigravid women had either acute or chronic PM infections compared with only 38% of multigravidae (Table 3). Clearly, primigravidae suffered from the burden of malaria throughout the course of pregnancy even when they received a high level of health care.

The malaria picture in multigravidae differed. They were rarely slide-positive (mean 1.5 ± 1.3 times out of 5.7 visits), had low peripheral parasitemias (Figure 3B), maintained low MoI (Figure 5A), developed anemia infrequently (Table 2), had asymptomatic infections, and only 38% had PM at delivery (Table 3). Parasites were not detected by microscopy in 21% multigravidae. On the other hand, essentially all (97%) multigravidae were PCR-positive, averaging 3.4 ± 1.8 PCR-positive prenatal visits during pregnancy, i.e., at 60% of their prenatal visits. Thus, multigravidae were frequently infected with *P. falciparum*, but were able to control their parasitemias to submicroscopic levels most of the time. Although the mean MoI detected each month was higher in primigravidae than multigravidae ($P = 0.0002$; Figure 5A), there was no difference in the total number of different parasite strains each woman harbored during the course of pregnancy (Table 1). This result suggests that both groups were infected with similar numbers

TABLE 3

Summary at delivery and pregnancy outcomes*

	Primigravidae (N = 6-9)	Multigravidae (N = 19-23)	P value
Length of gestation (wks)			
Peripheral blood	37.3 ± 4.1	38.9 ± 3.2	0.252
Blood smear positive (%)	57	17	0.037
PCR positive (%)	83	68	0.478
Mean MoI	3.3 ± 2.2	3.0 ± 1.5	0.905
Placental malaria positive (%)†	100	38	0.003
Mean placental parasitemia (%)	0.7 ± 0.5	0.10 ± 0.10	0.289
Anemia			
Mean hematocrit (% PCV)	34.3 ± 5.5	34.4 ± 3.4	0.571
Mean hemoglobin (gm/dL)	11.3 ± 1.1	11.4 ± 1.3	0.799
Women with anemia (%)‡	14.3%	4.0%	0.356
Placental pathology classification (%)			
Acute infections	50	19	0.030
Chronic infections	50	19	
Evidence of past infections	0	10	
No evidence of infection	0	52	
Mean birth weight (gm)§	2,908 ± 384	3,272 ± 484	0.054
Premature deliveries (%)	25	4	0.089
LBW babies (%)¶	13	5	0.486

* Mean ± SD.

† Based on impression smears plus histology.

‡ Hb < 10 gm/dL and PCV < 30%.

§ Twins were excluded.

¶ Low birth weight (LBW) is < 2,500 gm.

of genotypes, but that multigravidae cleared their parasites more rapidly. Even though multigravidae were frequently PCR-positive, only 38% had PM with low parasitemia (mean 0.1%) at delivery. Among these, 19% had acute infections suggesting they had become infected at the end of pregnancy, which agrees well with data on persistence of parasite genotypes (Figure 5B). The other 19% of infections were classified as chronic infections. It will be interesting to determine how the immune response differs between multigravidae who cleared their infections and the few who developed chronic placental malaria.

In high transmission areas, it is now well established that both young age and first pregnancy are risk factors for malaria.^{6,32–35} Pregnant women in the current study ranged from 14 to 38 years of age. In Ngali II, slide positivity in women in the general population declined over this age range from 43–28%, indicating that age is a factor in susceptibility to malaria (Figure 2). Unfortunately, the relative importance of age compared with gravidity could not be modeled by multivariate analysis, because all primigravidae were of a young age, i.e., the same women were included in both the group for young age and the group for primigravidae.

As previously reported, submicroscopic infections are common in pregnant women,^{19,21,22} as well as the presence of multiple parasite genotypes.^{19,22–24} The number of genotypes has been found to decrease with gravidity in some studies,^{23,24} but not in others.^{19,25} In Ngali II, women were bitten at least once every other day by an infected mosquito. It is therefore not surprising that the repertoire of multiple parasite genotypes changed constantly during pregnancy (Table 1). In general, a genotype would be present at one or two visits and then disappear. However, about 16% of the genotypes (19.4% based on *msp1* and 13.2% from *msp2* results) were detected from < 22 wk of pregnancy until term showing that a few genotypes persisted for a long period of time. Of particular interest was the finding of different MSP1-19 variants in primigravidae and multigravidae. The FUP and FVO variants of MSP-1 are the predominant forms in Ngali II, as well as in other countries.^{26,27} Although a small number of women was studied, results showed that young primigravidae were infected with the dominant types, whereas the majority of multigravidae were only infected with the other rare variants. Antibodies production against the variants is likely to be part of the “fine tuning” process of acquisition of immunity and would explain why young women were infected with the FUP and FVO forms, whereas more immune multigravidae eliminated these variants and only the rarer types were detected.

Weight gain during the second and third trimesters is reported to be smaller in African mothers than those in developed countries. Average second to third trimester weight gains of 4.6 and 6 kg have been reported in Tanzanian mothers and 5.8 kg for Kenyan women.^{36–38} Between the 14th and 35th wk, primigravidae and multigravidae in Ngali II gained $1.9 \pm 2.0.5$ and 6.5 ± 3.4 kg, respectively ($P = 0.009$). The influence of malaria on weight gain has not been adequately assessed. Unfortunately, the influence could not be accurately assessed in this study because all women were PCR-positive at multiple time points. A recent study in human immunodeficiency virus (HIV)-positive women showed that weight gain was lower in those who were slide-positive for malaria.³⁹ Because malaria increases the risk of anemia and LBW babies, it seems plausible it could also reduce maternal weight gain, thereby putting a higher burden on the health of young mothers.

Most *P. falciparum* infections in pregnant women remain asymptomatic. For example, Saute and others reported a prevalence of clinical malaria in 3% of 534 women in Mozambique.²⁵ Because symptoms of malaria are nonspecific; it is unclear how often pregnant women with mild infections develop malaria-associated symptoms. Many women in Ngali II reported symptoms consistent with malaria during pregnancy, but the prevalence of headache, fever, chills, and other symptoms were similar between blood smear positive and negative women. These results document that malaria-associated symptoms are rare in women living in hyperendemic areas.

In summary, longitudinal studies followed hematological, parasitological, and clinical changes in individual women who were repeatedly infected with *P. falciparum* during the course of pregnancy. The results re-emphasize the difference in disease severity between young primigravidae and older multigravidae with respect to frequency of slide positivity, anemia, and PM. However, all women in the study were repeatedly PCR-positive and infected with multiple parasite genotypes throughout pregnancy. The ability to control the level of parasitemia below a threshold level, not the absence of parasites, appears to be a key element in determining maternal health.

Received May 2, 2010. Accepted for publication July 22, 2010.

Acknowledgments: We express our profound gratitude to the chief and Elite of the village Ngali II for their support and the families who participated in the study. We are also grateful to the devoted physicians, research team members, entomologists, and village health workers who helped provide data included in this work.

Financial support: This study was supported by the NIAID grant no. U01 AI43888.

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