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The Hidden Burden of *Plasmodium vivax* Malaria in Pregnancy in the Amazon: An Observational Study in Northwestern Brazil

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Abstract. We measured the prevalence of malaria in pregnancy and estimated its impact on birth weight and length and maternal hemoglobin in 1,180 women from Juruá Valley, the main malaria hotspot in Brazil. Antenatal malaria episodes, 74.6% of them due to *Plasmodium vivax*, were microscopically diagnosed in 8.0% of the women and were associated with an average reduction in birth weight *z*-scores of 0.35 (95% confidence interval [CI] = 0.14–0.57) and in birth length *z*-scores of 0.31 (95% CI = 0.08–0.54), compared with malaria-free pregnancies. Affected mothers had a mean decrease in hemoglobin concentration at delivery of 0.33 g/100 mL (95% CI = 0.05–0.62 g/100 mL); 51.6% were anemic. The timing and frequency of antenatal infections influenced pregnancy outcomes and first- or second-trimester infections were not associated with decreased birth weight and length and maternal hemoglobin at delivery. Although repeated antenatal vivax infections were associated with poorer birth outcomes, even a single vivax malaria episode was associated with a significant reduction in birth weight and length and maternal hemoglobin. Overall, 7.5% women had the parasite's DNA found in peripheral blood at delivery. Most (83.1%) of these 89 perinatal infections were due to *P. vivax* and only 7.9% of them progressed to symptomatic disease after delivery. *Plasmodium vivax* and *Plasmodium falciparum* DNA was found in 0.6% and 0.3% of 637 cord blood samples examined, respectively, but only one newborn developed clinical neonatal malaria. Our results further challenge the notion that vivax malaria is relatively benign during pregnancy and call for better strategies for its prevention.

INTRODUCTION

More than 125 million pregnant women are globally at risk of malaria each year.[1] Malaria in pregnancy (MiP) is associated with a wide range of adverse outcomes for the mother, the fetus, and the neonate. In fact, stillbirth, miscarriage, low birth weight (LBW) caused by either intrauterine growth restriction or preterm birth, increased neonatal and maternal mortality, and reduced growth and neurocognitive function in early childhood are all well-known MiP complications.[2] The public health burden of MiP has been mostly investigated in malaria-endemic settings dominated by *Plasmodium falciparum* across Africa, Asia, and the Southwest Pacific.[2–4]

The clinical implications of malaria during pregnancy remain understudied in low-endemicity regions where *Plasmodium vivax* predominates, such as Latin America.[2,5,6] This is partially due to the fact that infections with *P. vivax* are believed to cause less severe clinical consequences in pregnant women than those with *P. falciparum*, although an increased risk of LBW and anemia associated with infection has been documented in large studies.[2,5,7–10] Erythrocytes parasitized with the former species do not sequester massively in the intervillous spaces as they do in *P. falciparum* infections,[11,12] but some MiP-associated histological changes in the placenta, such as syncytial knotting and increased thickness of the placental barrier, have been recently documented in Brazil[12,13] and may affect fetal nutrition and growth because of impaired transport and secretory functions.[14] Because primaquine (PQ) cannot be administered during pregnancy,[15] repeated MiP episodes due to *P. vivax* hypnozoite reactivation are relatively common and may adversely affect birth outcomes.

The 6,000–9,000 laboratory-confirmed MiP cases that are officially notified in Brazil each year, more than two-thirds of them caused by *P. vivax*, represent 4–6% of all malaria cases in the country.[16] More than 99% of these cases occur in the Amazon.[16] Intermittent preventive treatment during antenatal care visits is not recommended in this country, but pregnant women from malaria-endemic areas of Brazil must be screened for malaria parasites by conventional microscopy or rapid diagnostic tests at every antenatal care visit and receive supervised antimalarial treatment whenever infection is laboratory-confirmed.[15] However, antenatal care providers often fail to perceive MiP as a major preventable and treatable cause of morbidity in pregnant women and their offspring, and routine prenatal malaria testing, although formally recommended by the Ministry of Health, remains infrequent.[17] Moreover, available diagnostics often fail to detect peripheral parasitemias at delivery, either symptomatic or not, that are later diagnosed by more sensitive molecular methods,[18] further contributing to MiP underreporting.

Here, we investigated the burden of MiP in Juruá Valley, the main residual malaria hotspot in Brazil, where *P. vivax* is the dominant malaria parasite species. We combine microscopy-based diagnosis throughout pregnancy with molecular diagnosis at delivery to measure MiP prevalence and estimate its impact on fetal growth and maternal anemia in the largest Amazonian sample of malaria-exposed pregnant women thus far studied.

PATIENTS AND METHODS

Study site. Cruzeiro do Sul (07°37′S and 72°40′W), with 82,075 inhabitants, is the most populated municipality of

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Juruá Valley, northwestern Brazil. It currently accounts for 15.2% of the malaria burden countrywide, with 16,721 laboratory-confirmed cases in 2016—three-fourths due to *P. vivax* and one-fourth due to *P. falciparum*, with rare *Plasmodium malariae* infections (Ministry of Health of Brazil, unpublished data). Human-made vector breeding sites, mainly fish ponds opened for commercial aquaculture, are currently the major driver of residual malaria transmission in urban and periurban areas of Juruá Valley.[19] Malaria transmission occurs year-round and the main local malaria vector is the highly anthropophilic and mostly exophilic *Anopheles darlingi*,[20] although *Anopheles albitarsis* s.l. larvae are also abundant in both natural and human-made water bodies in the region.[21] Long-lasting insecticide-impregnated bed nets are estimated to be available in approximately one-third of households; periodic indoor spraying with residual insecticides is not consistently carried out in the municipality. The annual parasite index in Cruzeiro do Sul in 2016 was 231.9 malaria cases per 1,000 inhabitants (Ministry of Health of Brazil, unpub-lished data).

Study population and procedures. This prospective observational study was carried out at government-run antenatal care clinics and the only maternity hospital in Juruá Valley, where 96% of all deliveries in the municipality take place,[22] all located in the city of Cruzeiro do Sul. Pregnant women attending antenatal clinics or admitted to the maternity ward of the Women and Children's Hospital of Juruá Valley for delivery from July 2015 through June 2016 were eligible for inclusion. At enrollment, pregnant women were interviewed using a structured questionnaire. Data were entered into tablets programed with CSPro software (https://www.census.gov/programs-surveys/international-programs.html). Information on selected household assets was combined to derive a wealth index, used as a proxy of socioeconomic status for each participant.[23] The best estimate of gestational age was obtained from ultrasonography, carried out between 12 and 20 weeks of pregnancy and available for approximately one-third of study participants, or the reported date of the last menstrual period, for those without an ultrasound scan or with poor ultrasound images.[24] We used a portable SonoSite Titan machine (SonoSite, Bothell, WA) with curvilinear abdominal transducer, which was operated by two study radiologists. The following measurements were considered: crown-rump length (measured before week 14) or the biparietal diameter and femoral diaphysis length (measured between weeks 15 and 20). All images were reviewed by an expert obstetrician not involved in field work; 96.0% of them were scored as appropriate for pregnancy dating using a previously defined quality control protocol.[25] In a separate analysis, we found an excellent agreement between gestational age estimates obtained for the same study participants from ultrasonography and the reported date of the last menstrual period, with an average difference of 0.39 weeks (95% confidence interval [CI] = 0.27–0.50 weeks); Bland–Altman analysis revealed that 95% of the differences between these estimates are expected to lie in the interval between –2.3 and 3.1 weeks (B.H.L. and colleagues, manuscript in preparation).

Information regarding history of illnesses and treatments received during the current pregnancy was obtained through interview and review of clinical records. According to the latest guidelines of the Ministry of Health of Brazil, folate supplementation (5 mg/day) should be provided up to week 20 of pregnancy; at that time, oral iron supplementation (60 mg of iron/day) should be started. Anemia diagnosed in pregnant women is presumed to be associated with iron deficiency and treated with 120–240 mg of iron/day.[26] Data on antenatal malaria episodes diagnosed by thick-smear microscopy during pregnancy and up to 2 months after delivery were retrospectively obtained from the Malaria Epidemiological Surveillance and Information System database of the Ministry of Health of Brazil (http://200.214.130.44/sivep_malaria/). From this electronic database, we also recovered information on whether malaria episodes were correctly notified as MiP and treated properly. According to the latest malaria therapy guidelines of the Ministry of Health of Brazil,[27] primary *P. vivax* infections diagnosed in pregnant women by microscopy or rapid diagnostic tests are treated with chloroquine (CQ), total dose, 25 mg of base/kg over 3 days, without PQ because of the risk of hemolysis in the fetus.[16,27] To prevent *P. vivax* relapses, a weekly CQ dose of 300 mg over 12 weeks or until delivery is recommended but rarely prescribed. *Plasmodium falciparum* infections in the first trimester are treated with quinine (3 days) plus clindamycin (5 days), whereas those during the second and third trimesters are treated with a 3-day course of artemether (2–4 mg/kg/day) plus lumefantrine (12–24 mg/kg/day).[27] Malaria diagnosed by DNA amplification is not routinely treated, except if further confirmed by microscopy or an antigen-based rapid diagnostic test. At delivery, newborns were measured using an inextensible centimeter measuring tape accurate to the nearest millimeter, weighed on a digital scale accurate to the nearest gram, and examined by the attending clinician or obstetric nurse for any clinical abnormalities. *Z*-scores for birth weight and length were obtained using the INTERGROWTH-21st reference for gestational age and gender.[28] Venous blood samples (10 mL) were obtained from the mothers at the end of pregnancy (live-born delivery, stillbirth, or miscarriage) for complete blood counts and hemoglobin measurement as well as DNA extraction for molecular diagnosis of malaria, irrespective of any clinical symptoms. Umbilical cord blood samples (1 mL) were obtained for molecular diagnosis of congenital malaria.

Molecular diagnosis of malaria. We used a two-step strategy for molecular malaria diagnosis on venous and cord blood collected at delivery. We first screened samples with a genus-specific real-time polymerase chain reaction (RT-PCR) with a sensitivity of 2–5 parasites/μL of blood, followed by TaqMan assays (Applied Biosystems, Foster City, CA) for species-specific diagnosis, both methods targeting the *18S rRNA* gene of human malaria parasites.[29] DNA templates for polymerase chain reaction (PCR) amplification were isolated from 200 μL of whole blood using QIAamp DNA blood kits (Qiagen, Hilden, Germany), with a final DNA elution volume of 200 μL. The 20-μL reaction mixture of RT-PCR contained 5 μL of sample, 7.5 μL of 2 × Maxima SYBR Green quantitative PCR master mixture (Fermentas, Burlington, Canada), distilled water, and 0.1 μM of each oligonucleotide primer (forward, 5′-GTT AAG GGA GTG AAG ACG ATC AGA-3′ and reverse, 5′-AAC CCA AAG ACT TTG ATT TCT CAT AA-3′)[29] to amplify a 157- to 165-base pair fragment. We used a Step One Plus RT-PCR System (Applied Biosystems) for DNA amplification, with a template denaturation step at 95°C for 10 minutes (min), followed by 40 cycles of 15 seconds (sec) at 95°C, and 1 min at 60°C, with fluorescence acquisition at the end of each extension step. Amplification was immediately followed by a melting program consisting of 15 sec at 95°C, 1 min at 60°C,

and 95°C for 15 sec, with fluorescence acquisition at each temperature transition. For TaqMan assay,[29] each 20-μL reaction mixture contained 5 μL of sample DNA, 10 μL of TaqMan Universal Master Mix II (Applied Biosystems), 0.1 μM of each genus-specific unlabeled oligonucleotide primer, 0.08 μM of each species-specific labeled probe (*P. vivax*: 5′ VIC [Applied Biosystems proprietary green fluorescent dye]-AGC AAT CTA AGA ATA AAC TCC GAA GAG AAA ATT CT-QSY [Applied Biosystems proprietary quencher] 3′; *P. falciparum*: 5′ FAM [6-carboxyfluorescein]-AGC AAT CTA AAA GTC ACC TCG AAA GAT GAC T-QSY [Applied Biosystems proprietary quencher] 3′; *P. malariae*: 5′ NED [Applied Biosystems proprietary yellow fluorescent dye]-CTA TCT AAA AGA AAC ACT CAT-MGB [minor groove binder] quencher 3′), and distilled water to complete 20 μL. Polymerase chain reaction amplification comprised an initial step at 50°C for 2 min and template denaturation at 95°C for 10 min, followed by 45 cycles of 15 sec at 95°C and 1 min at 60°C. No-template controls (containing all reagents for amplification except for the DNA template) were run for every PCR microplate.

Clinical definitions. An antenatal malarial infection was defined as any episode of parasitemia, irrespective of the parasite density, diagnosed by thick-smear microscopy in study participants before delivery. Malaria is a notifiable disease in Brazil, with both laboratory diagnosis and treatment being available free of charge in government-run malaria outposts.[16] Because malaria treatment is not offered by private clinics and antimalarials cannot be purchased in local drugstores, we assume that virtually all antenatal malaria episodes in study participants were treated in public facilities and notified to the Ministry of Health. A perinatal malarial infection was defined when the parasite's DNA was detected by the research team on a venous blood sample collected at delivery, regardless of any symptoms. Congenital malaria was defined as any parasitemia detected by DNA amplification on a cord blood sample collected at delivery. Miscarriage was defined as a fetal death before 22 weeks of pregnancy; stillbirth was defined as a fetal death at or after 22 weeks of pregnancy. A delivery before 37 weeks of pregnancy was defined as preterm. Low birth weight was defined as a birth weight below 2,500 g, regardless of gestational age. Small-for-gestational-age (SGA) newborns are those whose weight is below the 10th percentile of the INTERGROWTH-21st reference for the gestational age.[28] Maternal anemia was defined as a hemoglobin concentration below 11 g/100 mL; hemoglobin levels below 7 g/100 mL characterized severe anemia.

Statistical analysis. Data were entered and cleaned using Stata 14.1 (StataCorp, College Station, TX) and analyzed with Stata 14.1 or SPSS 17.0 (SPSS, Inc., Chicago, IL). Proportions were compared by applying standard χ^2 tests to contingency tables and means were compared with standard unpaired Student *t* tests. Statistical significance was defined at the 5% level (two-tailed tests) and 95% CIs were estimated whenever appropriate.

Multiple logistic regression models were run to identify correlates of three outcomes: 1) antenatal malaria diagnosed by conventional microscopy, 2) perinatal malaria diagnosed by nucleic acid amplification, and 3) any MiP diagnosed during pregnancy or at delivery. Covariates included in the logistic models were age in years stratified into three categories (13–20, 21–30, and > 30 years), gravidity (0 = secundigravidae or multigravidae; 1 = primigravidae), years of schooling stratified into four categories (0, 1–5, 6–10, and > 10 years), wealth index stratified into quintiles in increasing order (first quintile, 20% poorest), area of residence (0 = urban, 1 = rural), and number of antenatal care visits attended (continuous variable). Demographic variables (age and gravidity) and those that were associated with the outcome at a significance level of at least 15% were retained in the final models, which were restricted to women without missing values. Separate models were built for any malaria and for specific malaria parasite species.

Multiple linear regression models were built to estimate the impact of malaria during pregnancy and malaria at delivery on three outcome variables: birth weight and birth length of newborns (described as *z*-scores for gestational age and gender) and maternal hemoglobin levels (in g/100 mL). This analysis was limited to pregnancies resulting in live-born singleton infants. Because the effects on health outcomes of sociodemographic determinants are often not direct, but mediated by more proximate factors, our modeling strategy considered distinct hierarchical levels of assumed causality.[30] Demographic and socioeconomic covariates, the most distal determinants, included age in years stratified into two categories (13–20 and > 20 years), gravidity (0 = secundigravidae or multigravidae; 1 = primigravidae), neonate's gender (0 = female, 1 = male), mother's years of schooling (continuous variable), wealth index (continuous variable), and area of residence (0 = urban, 1 = rural). The more proximate determinants included 1) environmental and behavioral factors such as prenatal smoking and prenatal alcohol use; 2) access to health care such as number of antenatal care visits attended (as a continuous variable), type of delivery (0 = vaginal, 1 = cesarean), and need for transfusion during pregnancy (only for the hemoglobin models); 3) self-reported comorbidities such as diabetes, chronic or gestational hypertension, and antenatal urinary tract infection; and 4) laboratory-confirmed antenatal or perinatal malaria. Unstandardized regression coefficients (*B*) were interpreted to indicate the influence of a given predictor on each outcome, while controlling for all other variables in the same or more distal hierarchical level. Separate regression models were built for each outcome. Demographic variables (age and gravidity) and those that were associated with the outcome at a significance level of at least 15% were retained in the final model, which only included women without missing values. Not unexpectedly, there was a strong association between age and gravidity (both entered into the models as dichotomic variables); young women were much more likely to be primigravidae ($\chi^2 = 238.13$, 1 d. f., $P < 0.0001$). We thus run separate models with either age or gravidity as the only demographic variable to confirm that a possible collinearity between them did not affect our estimates of the impact of malaria on birth outcomes and maternal anemia. Moreover, similar models were run using birth weight in *grams* and birth length in *centimeters*, instead of the respective *z*-scores for gestational age, as outcome variables. These models were additionally adjusted for gestational age.

Ethical approval. The institutional review board of the School of Public Health, University of São Paulo, approved the study protocol (no. 872.613, 2014). Written informed consent was obtained from all participants. Parents or guardians also gave written informed consent if the participants were minors (aged < 18 years).

RESULTS

Characteristics of study participants. From July 2015 through June 2016, 1,865 pregnant women attending antenatal clinics in the urban area of Cruzeiro do Sul, or admitted to the maternity ward of the Women and Children's Hospital of Juruá Valley for delivery, were invited to participate in this study. Of those, 1,538 women (82.5%) gave informed consent and were interviewed. Most women (63.8%) were enrolled at the maternity ward at the end of pregnancy, whereas 36.2% were enrolled at antenatal clinics. Compared with non-consenting subjects, enrolled women more often resided in urban areas (65.4% versus 58.3%, $P = 0.011$) and delivered live-born infants (97.3% versus 86.0%, $P < 0.001$). There were only 0.8% stillborn infants and 1.9% miscarriages among enrolled women, while 1.0% non-consenting women delivered stillborn infants, and 13.0% had miscarriages. The present analyses are limited to 1,180 study participants (76.7% of those enrolled) who had a blood sample collected at delivery and examined for malaria parasite's DNA (Figure 1). The vast majority (97.9%) of them attended at least one antenatal care visit, consistent with the high coverage (around 83%) of antenatal care previously reported in this municipality[31]; 56.5% attended seven or more visits. A comparison between study participants ($N = 1,180$) and those who were enrolled but had no blood sample collected at delivery is shown in Supplemental Table 1 (available online).

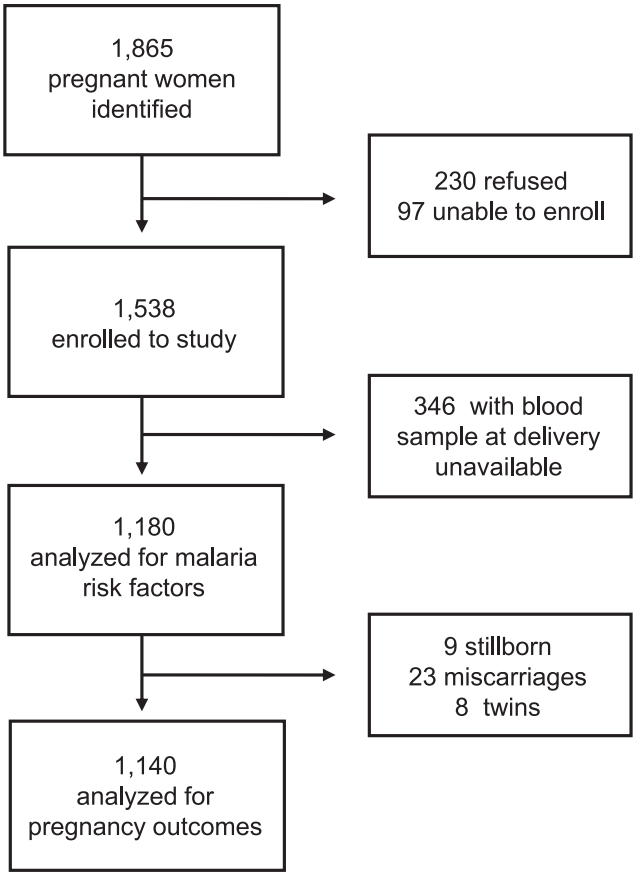

FIGURE 1. Study flow diagram. Between July 2015 and June 2016, 1,865 pregnant women attending antenatal clinics or admitted for delivery to the maternity ward of the Women and Children's Hospital of Juruá Valley, Cruzeiro do Sul (Brazil), were invited to participate. Reasons for exclusion and the final number of subjects analyzed are indicated.

Malaria in pregnancy and associated risk factors. Overall, 122 antenatal malaria episodes—29 (23.8%) due to *P. falciparum*, 91 (74.6%) to *P. vivax*, and 2 (1.6%) to both species—were diagnosed by conventional microcopy and treated in 94 (8.0%) study participants. Of these malaria episodes, 36 (29.5%), 40 (32.8%), and 46 (37.7%) occurred in the first, second, and third trimester of pregnancy, respectively. Twenty-eight (29.8%) mothers experiencing antenatal malaria had at least one laboratory-confirmed *P. falciparum* infection. Significantly, 37 (30.3%) antenatal malaria episodes were not correctly notified to the Ministry of Health as occurring in a pregnant woman; 24 (64.9%) of these misreported malarias were diagnosed in the first trimester of pregnancy, when some women might not be aware of their pregnancy status.

We detected malaria parasite's DNA in 89 (7.5%) mothers' peripheral blood samples collected at delivery, with 74 (83.1%) infections due to *P. vivax*, 14 (15.7%) due to *P. falciparum*, and one (1.1%) due to both species. No *P. malariae* infection was diagnosed by microcopy or DNA amplification. Interestingly, all perinatal infections were asymptomatic. Three (3.4%) asymptomatic parasite carriers had had a previous infection (one due to *P. vivax* and two due to *P. falciparum*) diagnosed and treated within two weeks before delivery, consistent with residual, posttreatment parasitemias. Seven (7.9%) women with asymptomatic perinatal infection, including one *P. vivax* carrier with antenatal malaria diagnosed shortly before delivery, later developed laboratory-confirmed clinical malaria within 2 months; all others remained free of symptoms and untreated over the 2-month postpartum follow-up. A total of 148 (12.5%) study participants had one or more MiP episodes, diagnosed either antenatally, at delivery, or both. Living in rural areas, the strongest predictor of MiP in our study population (Table 1), remained significantly associated with malaria after controlling for potential confounders, such as socioeconomic variables (Table 2). Interestingly, primigravidae were at increased risk of *P. falciparum* (but not of *P. vivax*) carriage at delivery, with borderline statistical significance (odds ratio = 4.802; 95% CI = 0.995–23.168; $P = 0.051$). However, multiple logistic regression analysis revealed no significant association between gravidity and risk of antenatal malaria of any type, vivax or falciparum.

Infant and maternal outcomes. Of the 1,180 study participants, 23 (1.9%) had a miscarriage, 9 (0.8%) had a stillbirth, and 1,148 (97.3%) delivered live-born infants, including 8 (0.7%) twin pairs. Three neonates (0.3%) died during hospitalization because of extreme prematurity; no mother died within 42 days of delivery. Among 1,140 singleton live-born infants analyzed, birth weights ranged between 780 and 5,060 g, with a mean of 3,222 g (standard deviation [SD], 509 g). The mean *z*-score for birth weight was 0.092 (SD, 1.026), ranging between –2.972 and 3.889 (Figure 2A). The mean *z*-score for length was 0.058 (SD, 1,083), ranging between –3.258 and 4.000 (Figure 2B; data for 1,132 neonates). Eighty-six (7.5%) live birth deliveries were preterm, 75 (6.6%) neonates had LBW, and 108 (9.5%) were SGA. Hemoglobin concentrations, measured in 1,101 mothers at delivery, ranged between 5.4 and 18.8 g/100 mL, with a mean of 11.1 (SD, 1.4) g/100 mL (Figure 2C). Overall, 442 (40.1%) mothers were anemic, but only 8 (0.7%) had severe anemia at delivery. Although iron and folate supplements are routinely prescribed to pregnant

TABLE 1

Unadjusted OR with respective 95% CI for associations between study participants' characteristics and antenatal or perinatal malaria in Cruzeiro do Sul, Brazil, 2015–2016

Characteristic	No. of subjects	Antenatal malaria (microscopy)		Perinatal malaria (DNA amplification)	
		OR (95% CI)	*P*	OR (95% CI)	*P*
Age (years)					
13–20	380	1.000 (reference)	–	1.000 (reference)	–
21–30	539	0.913 (0.579–1.439)	0.695	0.517 (0.325–0.821)	0.005
> 30	259	0.424 (0.212–0.849)	0.015	0.268 (0.128–0.558)	< 0.0001
Gravidity					
Primigravidae	458	0.930 (0.601–1.437)	0.743	1.599 (1.037–2.466)	0.034
Secundi- or multigravidae	722	1.000 (reference)	–	1.000 (reference)	–
Schooling (years)					
0	56	0.232 (0.031–1.719)	0.153	2.136 (0.848–5.377)	0.107
1–5	147	1.449 (0.778–2.698)	0.242	1.873 (0.966–3.633)	0.063
6–10	413	1.255 (0.789–1.996)	0.337	1.856 (1.132–3.042)	0.014
> 10	564	1.000 (reference)	–	1.000 (reference)	–
Wealth index (quintiles)					
1 (poorest)	240	1.000 (reference)	–	1.000 (reference)	–
2	240	0.880 (0.497–1.559)	0.662	0.572 (0.325–1.005)	0.052
3	219	0.557 (0.289–1.072)	0.080	0.357 (0.184–0.694)	0.002
4	246	0.527 (0.277–1.001)	0.050	0.265 (0.131–0.534)	< 0.0001
5 (least poor)	235	0.336 (0.160–0.709)	0.004	0.174 (0.076–0.399)	< 0.0001
Area of residence					
Urban	770	1.000 (reference)	–	1.000 (reference)	–
Rural	408	3.550 (2.293–5.496)	< 0.0001	4.931 (3.083–7.886)	< 0.0001
Number of antenatal care visits	1,175	1.009 (0.934–1.091)	0.816	0.928 (0.858–1.003)	0.061

CI = confidence intervals; OR = odds ratios.

women in Brazil, no attempt was made to evaluate adherence to supplementation among study participants.

Clinical impact of MiP. Microscopically diagnosed antenatal malaria, but not asymptomatic parasitemia at delivery, was a strong independent predictor of birth weight and length among singleton live-born infants (Table 3). Antenatal malaria was associated with an average reduction in *z*-scores for weight of 0.36 (95% CI = 0.14–0.57) and in *z*-scores for length of 0.31 (95% CI = 0.08–0.54). Quite similar impact estimates were derived from models that included either mother's age or gravidity (but not both) as covariates (data not shown). Separate multiple linear models estimated that infants born to

TABLE 2

Adjusted odds ratios and respective 95% CI, obtained by multiple logistic regression analysis, for associations between study participants' characteristics and malaria diagnosed during pregnancy (antenatal malaria) or at delivery (perinatal malaria) in Cruzeiro do Sul, Brazil, 2015–2016

Outcome	Covariate	aOR (95% CI)	*P*
Antenatal malaria	Schooling (years)		
	0	0.123 (0.016–0.964)	0.046
	1–5	0.835 (0.404–1.727)	0.626
	6–10	0.797 (0.466–1.364)	0.409
	> 10	1.000 (reference)	–
	Area of residence (rural vs. urban)	3.402 (2.127–5.441)	< 0.0001
Perinatal malaria	Age (years)		
	13–20	1.000 (reference)	–
	21–30	0.678 (0.387–1.187)	0.174
	> 30	0.393 (0.162–0.952)	0.039
	Wealth index (quintiles)		
	1 (poorest)	1.000 reference	–
	2	0.748 (0.409–1.370)	0.347
	3	0.512 (0.249–1.052)	0.068
	4	0.425 (0.193–0.937)	0.034
	5 (least poor)	0.373 (0.141–0.985)	0.047
	Area of residence (rural vs. urban)	3.942 (2.369–6.557)	< 0.001
Malaria in pregnancy (ante- or perinatal)	Age (years)		
	13–20	1.000 (reference)	–
	21–30	0.799 (0.507–1.258)	0.333
	> 30	0.470 (0.241–0.916)	0.027
	Wealth index (quintiles)		
	1 (poorest)	1.000 reference	–
	2	0.929 (0.569–1.516)	0.768
	3	0.485 (0.267–0.883)	0.018
	4	0.514 (0.280–0.947)	0.033
	5 (least poor)	0.389 (0.183–0.826)	0.014
	Area of residence (rural vs. urban)	3.575 (2.417–5.288)	< 0.0001

CI = confidence intervals; aOR = adjusted odds ratios. Complete information available for 1,175 subjects. Separate models were built for each outcome: antenatal malaria, perinatal malaria, and malaria in pregnancy (either antenatal, perinatal, or both).

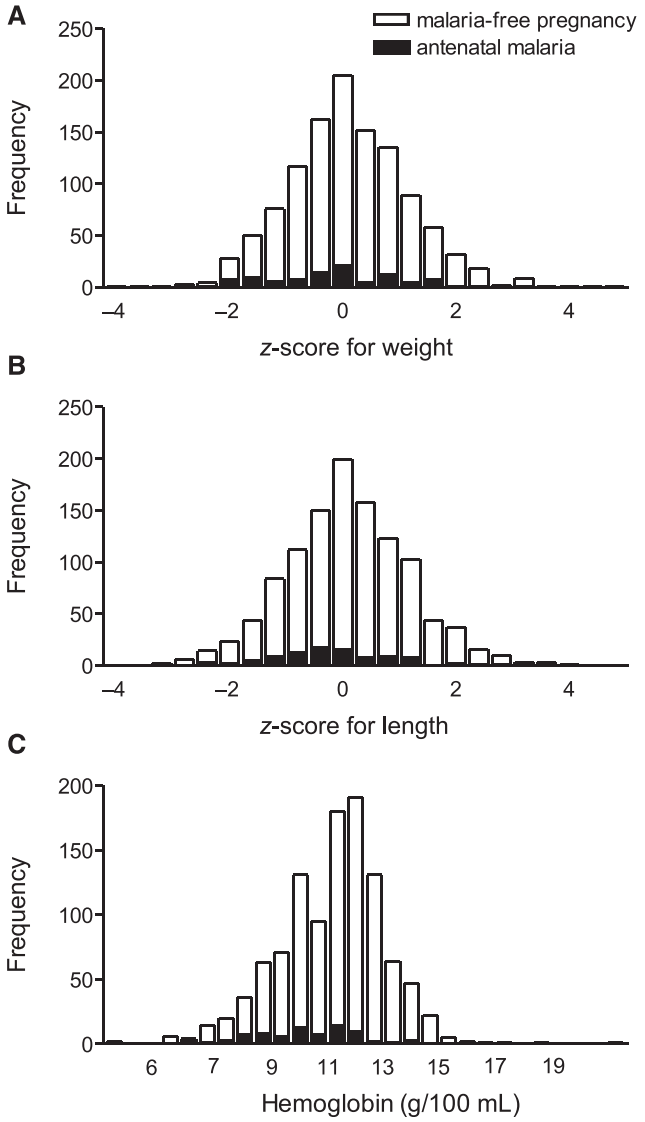

FIGURE 2. Frequency distribution of outcome measures—*z*-score for birth weight (**A**) and *z*-score for birth length (**B**) of live-born singleton infants, and maternal hemoglobin levels (g/100 mL) at delivery (**C**)—in Cruzeiro do Sul, Brazil, 2015–2016. Data for pregnancies with at least one antenatal malaria episode diagnosed by microscopy are indicated as black bar segments and those for malaria-free pregnancies with white bar segments. We analyzed 1,140 study subjects for birth weight, 1,132 for birth length (missing information for eight subjects), and 1,101 for hemoglobin concentrations (missing information for 39 subjects).

mothers who experienced one or more episodes of antenatal malaria were, on average, 0.12 g (95% CI = 0.04–0.20 g) lighter and 0.47 cm (95% CI = 0.05–0.88 cm) shorter, after controlling for gestational age and other potential confounders. Of 93 singleton neonates born to mothers experiencing one or more antenatal malarias, 3.2% were preterm, 8.6% had LBW, and 20.4% were SGA. Primigravidity, chronic hypertension (but not transient hypertension of pregnancy), and prenatal smoking were additional factors that negatively affected fetal growth (Table 3). Antenatal malaria (but not perinatal parasite carriage), cesarean delivery, and need for blood transfusion during pregnancy were independent predictors of decreased maternal hemoglobin levels at delivery (Table 3). Mothers with one or more antenatal malarias had a mean decrease in hemoglobin concentration of 0.33 g/100 mL (95% CI = 0.05–0.62 g/100 mL), after adjusting for potential confounders; 51.6% were anemic, but none had severe anemia.

We next ran a series of multiple linear regression models to explore how the frequency and timing of antenatal malaria episodes, as well as the infecting malaria parasite species diagnosed either ante- or perinatally, affected birth weight (Figure 3), birth length (Figure 4), and maternal hemoglobin levels (Figure 5), while controlling for the potential confounders listed in Table 3. The main findings may be summarized as follows: 1) even a single antenatal vivax malaria episode was significantly associated with reduced fetal growth and maternal hemoglobin, compared with malaria-free pregnancies; 2) repeated antenatal malarias of any type, experienced by 20 women, had a greater negative impact on birth weight (but not on birth length and maternal hemoglobin) than a single antenatal malaria episode; 3) the adverse effect of repeated malarias became more evident when the analysis was limited to *P. vivax* infections, which were associated with greatly reduced birth weight and hemoglobin levels at delivery; 4) third-trimester malaria episodes appeared to be more harmful than first- and second-trimester episodes—in fact, we were unable to detect a significant impact of first- and second-trimester infections on fetal growth or maternal hemoglobin evaluated at delivery; 5) the magnitude of the adverse impact on fetal growth and hemoglobin levels of third-trimester malarias remained similar, and significant, when we restricted the analysis to women experiencing a single antenatal infection in late pregnancy compared with those who remained malaria-free; and 6) although relatively little harm to the neonate and the mother could be associated with perinatal asymptomatic parasite carriage, *P. falciparum* infections at delivery were associated with a significant reduction in *z*-score for birth length.

Birth outcomes of infants putatively exposed to PQ in utero. Primaquine (0.5 mg of base/kg/day for 7 days) was retrospectively found to have been prescribed for treatment of 59 of 93 (63.4%) antenatal vivax or mixed-species malaria episodes diagnosed in 52 study participants. Five women had PQ prescribed for two episodes and one participant had PQ prescribed for three consecutive episodes. A large proportion (23% or 39.0%) of 59 PQ-treated infections were diagnosed and treated in the first trimester, but 15 (25.4%) and 21 (35.6%) PQ-treated infections were only diagnosed in the second and third trimester, respectively. Compliance with PQ prescription was not assessed in this study. Twenty-nine (49.1%) PQ-treated *P. vivax* infections had been correctly notified as MiP but were treated incorrectly. Interestingly, no adverse effect on birth outcomes was associated with putative exposure to PQ during pregnancy. All pregnant women given PQ prescription ($N = 52$) delivered live-born infants; their average *z*-scores for birth weight (–0.214 versus –0.089, $P = 0.725$) and birth length (–0.400 versus –0.080, $P = 0.299$) did not differ significantly, when compared with Student *t* tests, from those found in neonates from PQ-unexposed mothers who had at least one vivax malaria episode diagnosed and treated during this study.

Congenital malaria. Cord blood samples from 637 neonates were tested for the parasite's DNA. *Plasmodium vivax* and *P. falciparum* DNA was found in 4 (0.6%) and 2 (0.3%)

TABLE 3

Impact of antenatal and perinatal malaria with fetal growth and maternal hemoglobin concentration in Cruzeiro do Sul, Brazil, 2015–2016, after controlling for potential confounders by multiple linear regression analysis

Outcome	Model (no. of subjects)	Covariate	*B* (95% CI)	*P*
Birth weight (*z*-score)	1 (*n* = 1,139)	Antenatal malaria (yes vs. no)	–0.357 (–0.571 to –0.143)	0.001
		Gravidity (primi- vs. multigravidae)	–0.283 (–0.421 to –0.145)	0.001
		Type of delivery (cesarean vs. vaginal)	0.276 (0.155 to 0.397)	0.001
		Chronic hypertension (yes vs. no)	–0.250 (–0.435 to –0.066)	0.008
		Prenatal smoking (yes vs. no)	–0.278 (–0.482 to –0.075)	0.007
	2 (*n* = 1,139)	Perinatal malaria (yes vs. no)	–0.071 (–0.295 to 0.152)	0.532
		Gravidity (primi- vs. multigravidae)	–0.282 (–0.420 to –0.143)	< 0.001
		Type of delivery (cesarean vs. vaginal)	0.280 (0.158 to 0.402)	< 0.001
		Chronic hypertension (yes vs. no)	–0.245 (–0.430 to –0.059)	0.010
		Prenatal smoking (yes vs. no)	–0.267 (–0.471 to –0.063)	0.011
Birth length (*z*-score)	1 (*n* = 1,132)	Antenatal malaria (yes vs. no)	–0.311 (–0.541 to –0.082)	0.008
		Gravidity (primi- vs. multigravidae)	–0.242 (–0.391 to –0.092)	0.002
		Neonate's gender (male vs. female)	–0.179 (–0.305 to –0.052)	0.006
		Chronic hypertension (yes vs. no)	–0.231 (–0.434 to –0.028)	0.026
		Prenatal smoking (yes vs. no)	–0.299 (–0.520 to –0.078)	0.008
	2 (*n* = 1,132)	Perinatal malaria (yes vs. no)	–0.149 (–0.391 to 0.094)	0.229
		Gravidity (primi- vs. multigravidae)	–0.232 (–0.382 to –0.082)	0.002
		Neonate's gender (male vs. female)	–0.176 (–0.303 to –0.049)	0.007
		Chronic hypertension (yes vs. no)	–0.231 (–0.434 to –0.028)	0.026
		Prenatal smoking (yes vs. no)	–0.288 (–0.510 to –0.067)	0.011
Maternal hemoglobin (g/100 mL)	1 (*n* = 1,098)	Antenatal malaria (yes vs. no)	–0.335 (–0.624 to –0.047)	0.023
		No. of antenatal care visits attended	0.064 (0.032 to 0.097)	< 0.001
		Neonate's gender (male vs. female)	0.177 (0.018 to 0.336)	0.029
		Type of delivery (cesarean vs. vaginal)	–0.321 (–0.487 to –0.156)	< 0.001
		Pre- or perinatal blood transfusion (yes vs. no)	–3.284 (–4.215 to –2.352)	< 0.001
	2 (*n* = 1,098)	Perinatal malaria (yes vs. no)	–0.119 (–0.424 to 0.186)	0.443
		No. of antenatal care visits attended	0.063 (0.030 to 0.095)	< 0.001
		Neonate's gender (male vs. female)	0.181 (0.022 to 0.340)	0.026
		Type of delivery (cesarean vs. vaginal)	–0.328 (–0.495 to –0.161)	< 0.001
		Pre- or perinatal blood transfusion (yes vs. no)	–3.190 (–4.127 to 2.254)	< 0.001

CI = confidence intervals. Only significant ($P < 0.05$) associations between covariates other than malaria and birth and maternal outcomes are shown. Separate models were built for each outcome: antenatal malaria (model 1) and perinatal malaria (model 2).

cord blood samples, respectively. These infants were born to mothers found to carry exactly the same parasite species at the time of delivery. Only one of these congenital infections became symptomatic over the next 4 weeks, being confirmed by microscopic analysis of peripheral blood and treated within 20 days after birth.

DISCUSSION

Despite the accelerated progress toward malaria elimination in most of Latin America,[6,32] an estimated 4.3 million women are at risk of MiP each year in this continent.[1] Here, we show that antenatal malaria remains common in the main malaria hotspot of Brazil, affecting 8.0% of our study participants—comparable with the recent prevalence estimate at 7.9% in Guayaramerín, an endemic setting in Bolivia next to the border with Brazil.[5] Neither age nor gravidity emerged as a significant predictor of risk for antenatal malaria in our study, after controlling for potential confounders. Of note, nearly one-third of the antenatal malarial infections were not correctly notified as occurring in pregnant women, implying that MiP may be substantially underreported in Brazil. Moreover, we found further evidence that health-care providers often fail to comply with the country's malaria treatment guidelines for pregnant women in Brazil.[17] We retrospectively found that PQ was prescribed for more than half of the antenatal *P. vivax* infections diagnosed during the study, not only in the first trimester (when the pregnancy status might be unknown) but also in infections correctly reported as MiP. This may expose the fetus to severe hemolysis risk. However, we found no evidence of adverse birth outcomes in neonates putatively exposed in utero to PQ.

The vast majority of maternal malaria infections detected by DNA amplification at delivery remained asymptomatic, undiagnosed, and untreated up to 42 days after delivery. Whether asymptomatic perinatal infections are harmful to the mother and the neonate in this setting remains unclear; we only found a statistically significant association between falciparum (but not vivax) infection at delivery and decreased birth length (Figure 4). Available prevalence estimates of perinatal malaria in Latin America, few of them from population-based surveys, range widely between 3.5% in the city of Manaus, Brazil,[10] and 39.0% in northwestern Colombia.[33] Congenital infection, found in 0.9% of our cord blood samples tested for malarial DNA, has also recently been reported in Guatemala (14.8% of DNA-positive cord blood samples),[10] northwestern Colombia (3.0–13.0%),[10,34] and Manaus, Brazil (1.0%).[10] Severe and complicated cases of neonatal malaria have been reported in Colombia,[35,36] suggesting that at least some of these subpatent and asymptomatic perinatal infections, detected by molecular methods, may later progress to a full-blown disease in similar endemic settings.

Infection with *P. vivax*, the dominating species in Latin America, may impair fetal growth and cause anemia in pregnant women.[5,7–10,37,38] However, currently available evidence came mostly, although not exclusively,[38] from studies that detected parasitemias at delivery, rather than throughout the antenatal period. We thus examined the impact of the frequency and timing of *P. vivax* malaria episodes during

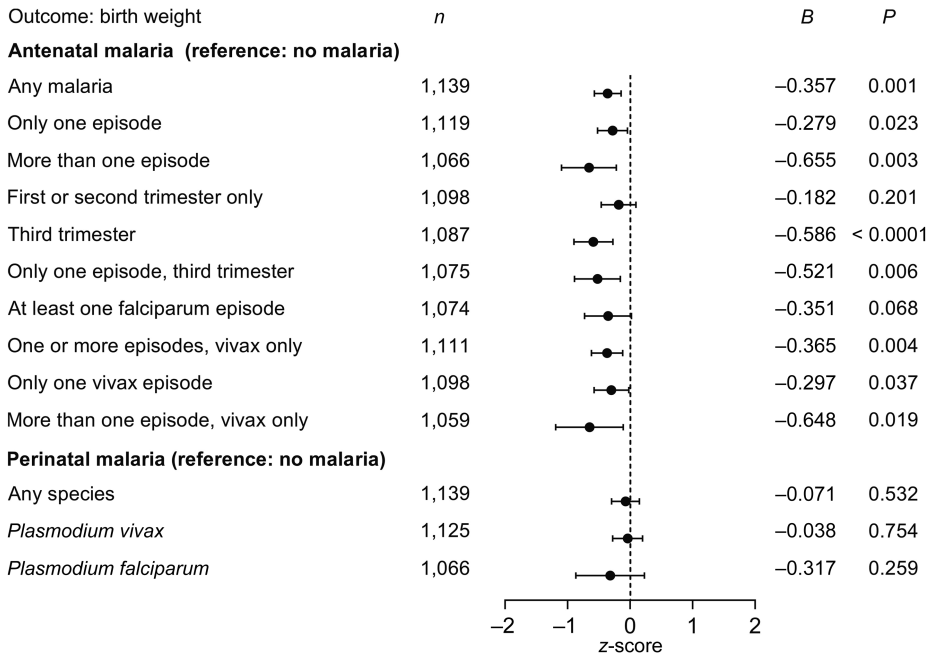

FIGURE 3. Impact of number, timing, and species of antenatal and perinatal malaria episodes on birth weight *z*-score in Cruzeiro do Sul, Brazil, 2015–2016, as determined by multiple linear regression analysis. The unstandardized regression coefficients (*B*) were interpreted to indicate the average change in birth weight *z*-score attributable to each malaria type, frequency, or timing throughout the pregnancy, compared with no malaria, while controlling for the potential confounders listed in Table 3. Note that each *B* estimate and respective 95% confidence interval and *P* value were derived from a separate model, which included different numbers of study participants (*n*). For example, in the comparison between participants with multiple antenatal malaria episodes (*N* = 20) with those with antenatal malaria (*N* = 1,046), 73 subjects with a single malaria episode were excluded from the analysis.

pregnancy on fetal growth and maternal hemoglobin in a low-endemicity setting. We observed significantly lower birth weights (mean *z*-score reduction of 0.30) and lengths (mean *z*-score reduction of 0.41), as well as lower maternal hemoglobin concentrations (mean reduction of 0.40 g/100 mL), in pregnancies with a single antenatal episode of vivax malaria, compared with malaria-free pregnancies, showing that one vivax malaria may suffice to adversely affect pregnancy

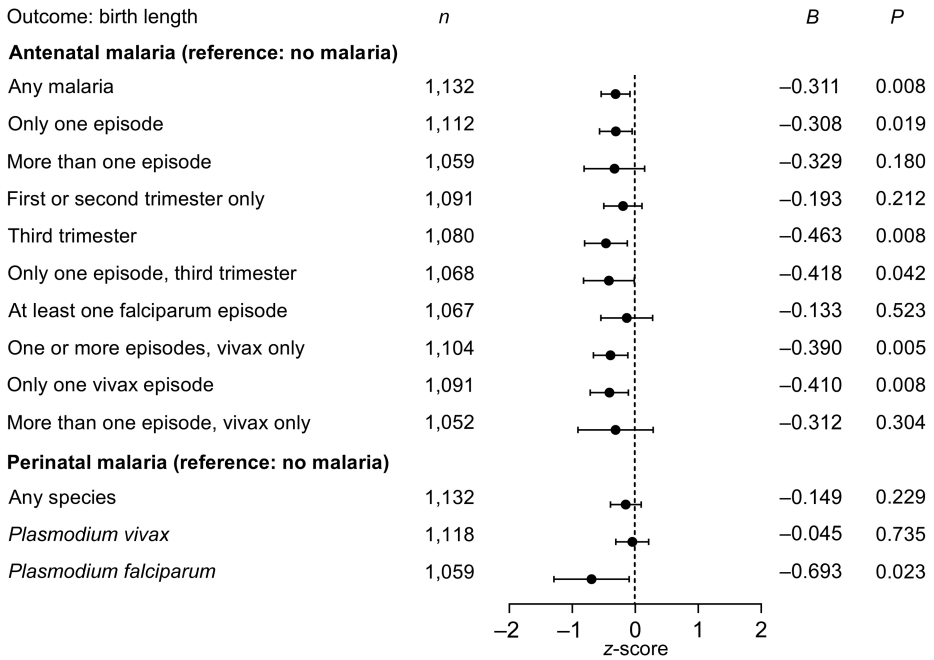

FIGURE 4. Impact of number, timing, and species of antenatal and perinatal malaria episodes on birth length *z*-score in Cruzeiro do Sul, Brazil, 2015–2016, as determined by multiple linear regression analysis. The unstandardized regression coefficients (*B*) were interpreted to indicate the average change in birth length *z*-score attributable to each malaria type, frequency, or timing throughout the pregnancy, compared with no malaria, while controlling for the potential confounders listed in Table 3. Note that each *B* estimate and respective *P* value were derived from a separate model, which included different numbers of study participants (*n*), as explained in the legend of Figure 3.

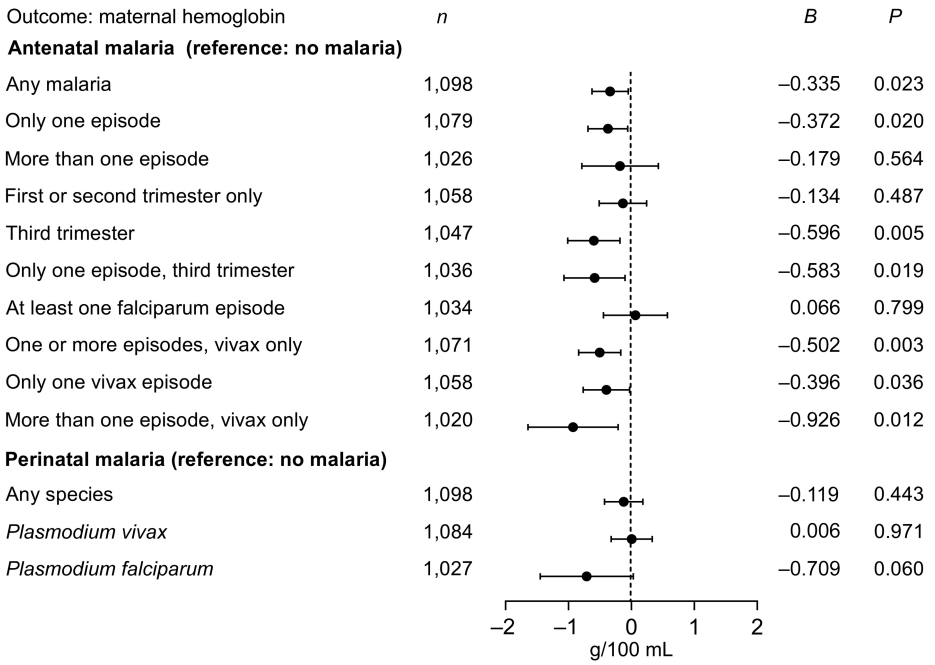

FIGURE 5. Impact of number, timing, and species of antenatal and perinatal malaria episodes on maternal hemoglobin concentration at delivery in Cruzeiro do Sul, Brazil, 2015–2016, as determined by multiple linear regression analysis. The unstandardized regression coefficients (*B*) were interpreted to indicate the average change in hemoglobin concentration (g/100 mL) attributable to each malaria type, frequency, or timing throughout the pregnancy, compared with no malaria, while controlling for the potential confounders listed in Table 3. Note that each *B* estimate and respective 95% confidence interval and *P* value were derived from a separate model, which included different numbers of study participants (*n*), as explained in the legend of Figure 3.

outcomes. Repeated infections, many of them likely to originate from relapsing *P. vivax* parasites, were associated with poorer outcomes. In fact, the average decrease in birth weight *z*-score (0.65) and maternal hemoglobin (0.93 g/100 mL) was greater among women with recurring malarias during pregnancy, consistent with a cumulative adverse impact of these repeated antenatal infections.

We failed to observe a significant adverse impact of early antenatal malaria episodes, namely those occurring in the first or second trimester of pregnancy. In this and another recent study,[38] only third-trimester *P. vivax* infections appeared to affect fetal growth and maternal hemoglobin levels evaluated at delivery. Another large study has shown an increased proportion of SGA infants born to mothers who had vivax malaria after 20 weeks' pregnancy, compared with those with no MiP.[39] By contrast, falciparum malaria before 20 weeks' pregnancy has been clearly shown to affect fetal growth measured by repeated ultrasound scans before delivery[40,41] and can cause LBW and maternal anemia at delivery in some African settings.[41] Even a single episode of either *P. vivax* or *P. falciparum* malaria diagnosed and successfully treated during the first trimester was found to increase the risk of miscarriage, although not that of LBW, in a large cohort in Thailand.[38] As previously suggested,[38] these findings are consistent with a relatively efficient growth recovery, over the remaining gestation, by fetuses that survived an early in utero exposure to malaria, provided that antenatal infections are properly diagnosed and treated.

The present study has some limitations. First, women whose pregnancies ended as miscarriage or stillbirth were more likely to be excluded from our study population. This limited our statistical power to analyze the impact of MiP on miscarriage and stillbirth.[38,42] Because we analyzed pregnancy outcomes only for live births, a survivorship bias may have affected our analyses of the impact of early-pregnancy infections. Accordingly, one can argue that pregnancies most severely affected by early malaria episodes were more likely to end as miscarriage or stillbirth, being excluded from our analysis. Second, data on antenatal malaria episodes were retrieved retrospectively and no blood samples were available for further confirmatory diagnostic tests. Because routine antenatal malaria screening, although recommended,[16] has not been widely implemented across the Amazon Basin of Brazil,[17] nearly all malarial infections diagnosed and treated during pregnancy had been identified passively, when febrile women sought treatment in malaria outposts. This precludes any analysis of the impact of antenatal episodes of asymptomatic parasitemia on birth outcomes in this population. Third, parasite's DNA detection in the peripheral blood was the only diagnostic technique performed at delivery and its detection threshold (2–5 parasites/μL) is relatively low. No placental samples were available for histopathological examination. Molecular methods on peripheral blood samples may be more sensitive for perinatal malaria diagnosis than microscopic diagnosis or antigen detection on peripheral blood or placenta samples,[17] but placental histopathology can additionally help to determine the timing and intensity of infection and its associated inflammatory changes.[11,12] A careful association between histological changes in the placenta and birth outcomes in vivax malaria might contribute to our understanding on the pathophysiology of MiP caused by a parasite species that does not sequester massively but significantly affects placental functions. Finally, the infrequency of *P. falciparum* precludes further between-species comparisons

of the impact of MiP on pregnancy outcomes in this low-endemicity setting.

In conclusion, our findings further challenge the common notion that vivax malaria during pregnancy, contrary to falciparum malaria, is a relatively benign health condition in low-endemicity countries. Antenatal malaria infections in the third trimester of pregnancy are associated with significant fetal growth impairment and lower maternal hemoglobin levels at delivery. Such adverse outcomes can be observed even in mothers experiencing a single antenatal *P. vivax* infection but become more evident in those with repeated vivax malaria episodes throughout pregnancy. Moreover, compliance with the national guidelines for vivax malaria treatment in pregnancy is poor, leading mothers and their fetuses to be often exposed to PQ. In particular, better ways to prevent *P. vivax* relapses in pregnancy are urgently needed because the currently recommended weekly CQ prophylaxis for recurring infections[15] is rarely prescribed by attending health professionals and very likely to be poorly adhered to by patients. These results call for improved strategies for MiP prevention in areas where intermittent preventive treatment is not feasible.

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The following are supplemental materials and will be published online only

SUPPLEMENTAL TABLE 1

Comparison between study participants included in the present analyses and those who were eligible but did not provide blood samples, Cruzeiro do Sul, Brazil, 2015–2016

Characteristic	Enrolled to study (no. subjects with information)	Not enrolled to study (no. of subjects with information)	P (χ^2)
Age	(n = 1,178)	(n = 327)	0.589
13–20	380	96	–
21–30	539	154	–
> 30	259	77	–
Wealth index (quintiles)	(n = 1,180)	(n = 346)	–
1 (poorest)	240	66	0.112
2	240	65	–
3	219	86	–
4	246	60	–
5 (least poor)	235	69	–
Area of residence	(n = 1,178)	(n = 327)	0.370
Urban	770	205	–
Rural	408	122	–
Gravidity	(n = 1,180)	(n = 346)	0.301
Primigravidae	458	145	–
Multigravidae	722	201	–
Neonate's gender	(n = 1,149)	(n = 341)	0.214
Female	576	184	–
Male	573	157	–
Twins	(n = 1,157)	(n = 343)	0.829
Yes	8	2	–
No	1,149	341	–
Prenatal smoking	(n = 1,180)	(n = 346)	–
No	1,128	330	0.863
Yes	52	16	–
Alcohol use	(n = 1,180)	(n = 346)	0.049
Yes	206	45	–
No	974	301	–
Urinary tract infection	(n = 1,178)	(n = 327)	0.139
Yes	762	197	–
No	416	130	–
Diabetes	(n = 1,180)	(n = 346)	0.684
Yes	20	7	–
No	1,160	339	–
Chronic hypertension	(n = 1,180)	(n = 346)	0.074
Yes	132	51	–
No	1,048	295	–
Antenatal malaria	(n = 1,180)	(n = 346)	–
Any species	94	23	0.418
Plasmodium falciparum	28	9	0.808
Plasmodium vivax	66	14	0.256
Number of antenatal malarias	(n = 1,180)	(n = 346)	0.486
0	1,086	323	–
1	74	16	–
> 1	20	7	–
Type of delivery	(n = 1,157)	(n = 343)	0.429
Vaginal	652	185	–
Cesarean	505	158	–
Pregnancy outcome	(n = 1,180)	(n = 346)	0.386
Live-born	1,148	340	–
Stillborn	9	3	–
Miscarriage	23	3	–
Low birth weight	(n = 1,148)	(n = 339)	0.102
Yes	78	32	–
No	1,070	307	–