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Circulating Cytokines Associated with Poor Pregnancy Outcomes in Beninese Exposed to Infection with *Plasmodium falciparum*

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ABSTRACT Malaria during pregnancy is a major cause of maternal morbidity as well as fetal and neonatal mortality. Previous studies, including our own, suggested that placental and peripheral cytokine and chemokine levels measured at delivery can be used as biomarkers for pregnancy outcomes. However, the timing of malaria infection during pregnancy matters, and these studies do not address the effect of different cytokines in peripheral blood plasma samples taken at early and midpregnancy and at delivery. Here, we aimed to investigate whether peripheral plasma cytokine levels were associated with pregnancy outcomes in a cohort of 400 Beninese pregnant women. Using a high-sensitivity cytometry-based method, we quantified the levels of interleukin-4 (IL-4), IL-5, IL-10, IL-12p70, and gamma interferon (IFN-γ) in peripheral plasma samples taken at two time points during pregnancy and at delivery in various groups of pregnant women identified with *Plasmodium falciparum* infection, with anemia, with preterm births, or giving birth to babies who are small for their gestational age. We found that, consistently at all time points, elevated levels of IL-10 were strongly and significantly associated with *P. falciparum* infection, while the levels of IFN-γ at inclusion and delivery were weakly but also significantly associated. Low levels of IL-5 at delivery were associated with a greater risk of both preterm births and small-for-gestational-age babies. The immunosuppressive effects of IL-10 likely affect the overall cytokine equilibrium during pregnancy in women harboring *P. falciparum* infections. Our findings highlight the peripheral signature of pregnancy outcomes and strengthen the idea of using cytokines as diagnostic or prognostic markers.

KEYWORDS cytokine, malaria, pregnancy outcomes

According to the World Health Organization, an estimated 228 million cases of malaria occurred worldwide, with 405,000 deaths, in 2018 (1). *Plasmodium falciparum* accounted for the majority of cases. In areas with high transmission of malaria, children under 5 years and pregnant women are particularly susceptible to infection, illness, and death (1). In sub-Saharan Africa, 11 million pregnant women exposed to malaria infections in 2018 would have delivered about 872,000 children with low birth weight (1). The increased susceptibility of pregnant women to malaria compared to nonpregnant adults is due, in part, to the physiological adaptation of the maternal immune system to prevent rejection of the fetus as a semiallogeneic transplant (2). In this context, an important role is played by cytokines, the levels of which change during the different trimesters of pregnancy.

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During the first trimester, proinflammatory cytokines act as mediators of the embryo-maternal paracrine dialogue associated with apposition, attachment, and invasion (3, 4). In this process, members of the interleukin-6 (IL-6) cytokine superfamily are required at the early implantation stage. While leukemia inhibitory factor (LIF) is expressed at the highest concentrations in the endometrial glands and is strongly associated with normal implantation, IL-11 acts on endometrium luminal epithelium to facilitate blastocyst attachment and implantation (5, 6). The IL-12/IL-15/IL-18 system cooperates with the IL-1 system and other cytokines, such as vascular endothelial growth factor (VEGF) and epidermal growth factor (EGF), via interactions with endometrial leukocytes and natural killer cells in local angiogenesis and tissue remodeling (7). Cellular immune responses also play an important role with human decidual natural killer cells that promote trophoblast invasion through the production of IL-8, interferon (IFN)-inducible protein 10 (IP-10), VEGF, and placental growth factor (8).

Once implantation has taken place, successful pregnancy is characterized by a cytokine balance that is tipped toward a predominantly T helper 2 (Th2)-type response. Progesterone contributes to support IL-3, IL-4, IL-5, and IL-10 production, which inhibits Th1 responses and favors allograft tolerance in women (9). In addition, in the systemic circulation of healthy pregnant women, IL-4, IL-6, IL-10, and IL-13 production progressively increases, while the serum levels of most Th1-type cytokines decrease significantly after the second trimester compared with the levels observed during the first trimester (10).

Given the importance of the timely regulation of cytokine networks during pregnancy, the dysregulation of this system at both local and systemic levels is invariably characteristic of adverse pregnancy outcomes, including spontaneous abortion, preterm labor, pre-eclampsia, and intrauterine growth restriction (IUGR) (11, 12). Thus, Th1-type response alterations at implantation sites have been associated with placental tissue damage in some inflammatory diseases. Such is the case when *P. falciparum* infection occurs during pregnancy, leading to a pronounced proinflammatory response in the placenta with deleterious effects on both maternal and fetal health (13, 14).

So-called placental malaria (PM) is characterized by the sequestration of parasite-infected erythrocytes in the maternal intervillous blood spaces of the placenta. The parasite-derived protein VAR2CSA, which is transported to and expressed on the surface of infected erythrocytes, mediates this sequestration (15). VAR2CSA binds to chondroitin sulfate A (CSA) on the proteoglycan syndecan-1, which is expressed by syncytiotrophoblasts (16). The infected erythrocytes that cause PM elicit Th1-type cytokine production in the placenta, where elevated levels of proinflammatory cytokines, such as tumor necrosis factor alpha (TNF-α), IL-2, IFN-γ, and IL-1β, are observed, especially in primigravidae (17). Increased placental levels of chemokines have also been described, leading to monocyte infiltration along with the recruitment of macrophages, cytotoxic T cells, B cells, and granulocytes into the placenta (18, 19). T cell proliferation and enhanced macrophage phagocytic activities, aimed at controlling parasite proliferation, are a feature of the inflammatory response during PM (17, 20).

The persistence of infected erythrocytes, accompanied by the excessive accumulation of leukocytes in the intervillous spaces of the placenta, are ultimately responsible for the pathological outcomes of PM. In this context, numerous studies have investigated the relationships between cytokine production during PM and pregnancy outcomes (21–25). High levels of placental TNF-α were associated with several pregnancy outcomes, including severe anemia, low birth weight (LBW), and IUGR (21). Likewise, high expression of IL-8 associated with IUGR was found in placentas of multigravidae (21, 22). Furthermore, in a previous study, our findings revealed an association between babies with low birth weight for their gestational age (GA) and low placental plasma levels of IFN-γ and IL-5, independent of gravidity (25).

However, in peripheral blood, parasite-specific immune responses may be decreased due to the trafficking of cells out of the circulation and the reduction of peripheral parasitemia as the development of the placenta is completed (26–28). Low levels of cytokines were detected in systemic plasma of pregnant women at delivery,

TABLE 1 Description of the study population

Characteristic	Value
Women	
Mean age (yr)	26.5 (25.9–27.1)[a]
Mean gestational age at inclusion[b] in days (range)	114 (38–189)
Mean gestational age at ANV4[b] in days (range)	241 (164–285)
Mean no. of ANV (range)	4 (1–7)
Mean no. of IPT[c] (range)	1.94 (0–3)
No. (%) with:	
Primigravidity	64 (16)
Use of bed nets	88 (22)
Absence of education	244 (61)
Malaria[d] (no. with *P. falciparum* infection/total no. tested)	
At inclusion	70/397 (18)
During follow-up	
0	227/397 (57)
1	112/397 (28)
≥2	58/397 (15)
Delivery	59/388 (15)
Anemia[e] (no. with anemia/total no. tested)	
At inclusion	120/393 (31)
During follow-up	
0	105/369 (28)
1–3	47/369 (13)
≥4	217/369 (59)
Delivery	168/373 (45)
Children	
Gestational age at birth, mean days (range)	277 (227–300)
No. of preterm births[f]/total no. tested (%)	20/359 (6)
Birth weight	
Mean (g)	2,979 (2,934–3,023)[a]
No. with SGA[g]/total no. tested (%)	64/391 (16)

[a]Represents 95% confidence intervals.
[b]Ultrasound examination was performed before the 24th week of gestation.
[c]Intermittent preventive treatment.
[d]On the basis of thick blood smear microscopy.
[e]Hemoglobin concentration, <10 g/dl.
[f]Babies born before 37 weeks of gestation.
[g]According to the charts of Schmiegelow et al. (51).

and these were not significantly related to pregnancy outcomes (23, 24, 29). Immune responses in the context of malaria during pregnancy are generally underexplored, and longitudinal studies could lead to a better understanding of the relationship between peripheral immune markers and poor pregnancy outcomes.

Here, the relationships between *P. falciparum* infections and peripheral plasma cytokine activity during pregnancy, on one hand, and the consequences of different immune equilibria on pregnancy outcomes, on the other hand, were investigated. Therefore, circulating plasma levels of IL-4, IL-5, IL-10, IL-12p70, and IFN-γ were measured at different gestational ages in a cohort of Beninese women to identify biomarkers potentially of prognostic and/or diagnostic value for pregnancy-associated malaria (PAM) and/or for poor pregnancy outcomes.

RESULTS

Description of the study population. From the 1,037 women included in the whole STOPPAM (Strategies to Prevent Pregnancy-Associated Malaria) cohort, 400 were selected according to their clinical and parasitological status for the current study. The characteristics of the women and their newborns are presented in Table 1. The women's mean age was 26.5 years (95% confidence interval [CI], 25.9 to 27.1), 64 (16%) were primigravid, and the mean gestational age (GA) assessed by ultrasound at inclusion was 114 days (16.3 weeks). For comparison, the mean age of the whole cohort was 26.4 years, 18.2% were primigravidae, and the mean GA at inclusion was 17.2 weeks (30). At inclusion in this subgroup of 400, 70 (18%) women were infected

with *P. falciparum* and 120 (31%) were anemic. During the follow-up, the mean GA at antenatal visit 4 (ANV4) was 34.4 weeks, slightly more than half (57%) remained free of *P. falciparum* infection, while 112 (28%) were infected once and 58 (15%) were infected twice or more. Anemia was detected in 105 (28%) throughout follow-up, while 217 (59%) were found to be anemic on more than four occasions. At delivery, 59 (15%) mothers were infected with *P. falciparum* and 168 (45%) were anemic. Mean GA at delivery was 277 days (39.6 weeks; 95% CI, 276 to 278), while 20 (6%) babies were preterm births (PTB) and 64 (16%) were small for their gestational age (SGA).

Pattern of peripheral plasma cytokine concentrations during pregnancy. We assessed the levels of IL-4, IL-5, IL-10, IL-12p70, and IFN-γ at inclusion, at ANV4, and at delivery. Figure 1 and Table 2 show the overall patterns observed in univariate analyses without segregation of women into different groups. The concentration of IL-10 decreased at ANV4 and increased at delivery ($P = 0.015$), while the levels of the other cytokines showed a marked tendency to decline during pregnancy, significantly so in the case of both IL-5 and IL-12p70 ($P < 0.001$ and $P = 0.043$).

Table 3 shows the results of univariate comparison of cytokine levels during pregnancy between the healthy control group and those with infection, with anemia, or with poor pregnancy outcomes. IL-10 was higher in all groups (PAM, anemia, PTB, and SGA) at all times (inclusion [INC], ANV4, and delivery [DEL]) compared to the control group.

Table 4 shows the cytokine distribution as a function of the number of *P. falciparum* infections detected during pregnancy (none, one, and two or more, but excluding those detected at delivery). The levels of IL-10 were significantly higher in the infected versus uninfected groups, regardless of the number of infections or the time point ($P < 0.001$ in all cases) but with a tendency for levels to decrease over the course of pregnancy among women with two or more infections. The median levels of the other cytokines did not differ among the different groups at the different time points.

Association between cytokine levels, *P. falciparum* infection, and pregnancy outcomes using multivariate analysis. (i) Association between *P. falciparum* infections and cytokine levels at each time point. Table 5 shows the results of multivariate analyses performed to determine the influence of *P. falciparum* infection at each time point on cytokine concentrations at the same time after adjustment for anemia, PTB, and SGA at each time point during pregnancy. Among *P. falciparum*-infected mothers, IFN-γ concentrations at inclusion ($P = 0.032$; average variation of cytokine concentrations associated with the presence of *P. falciparum* infection [coef], 0.43) and at delivery ($P = 0.004$, coef = 0.67) were slightly but significantly increased, whereas IL-10 levels were strongly and significantly increased at all three time points (INC, $P < 0.001$, coef = 11.19; ANV4, $P < 0.001$, coef = 29.01; DEL, $P < 0.001$ coef = 45.95). Of note, IL-10 levels were lower in multigravid than primigravid women at inclusion ($P = 0.004$, coef = -4.43) and ANV4 ($P = 0.006$, coef = -11.33).

(ii) Association between *P. falciparum* infections during pregnancy and cytokine levels at delivery. After adjusting for anemia, PTB, SGA, and *P. falciparum* infection at delivery, the association between the occurrence of PAM and cytokine levels at delivery was evaluated (Table 5). *P. falciparum* infection was associated only with IL-10 concentrations: women who had had at least one *P. falciparum* infection during pregnancy had a significantly higher level of IL-10 at delivery than those with no infection during pregnancy ($P = 0.036$, coef = 24.01). Also of note, the level of IL-10 was significantly lower as a function of increased numbers of *P. falciparum* infections during pregnancy ($P = 0.017$, coef = -13.83).

(iii) Association between cytokine concentrations at inclusion and *P. falciparum* infection status during pregnancy. The association between cytokine levels at inclusion and the occurrence of *P. falciparum* infection during pregnancy was analyzed (Table 6), controlling for PTB, SGA, and anemia and adjusting for cytokine levels on *P. falciparum* infection at inclusion. Elevated IFN-γ concentrations at inclusion were associated with a significantly reduced risk of *P. falciparum* infection during pregnancy ($P = 0.035$, odds ratio [OR] = 0.82), while for increased IL-12p70 levels the risk of

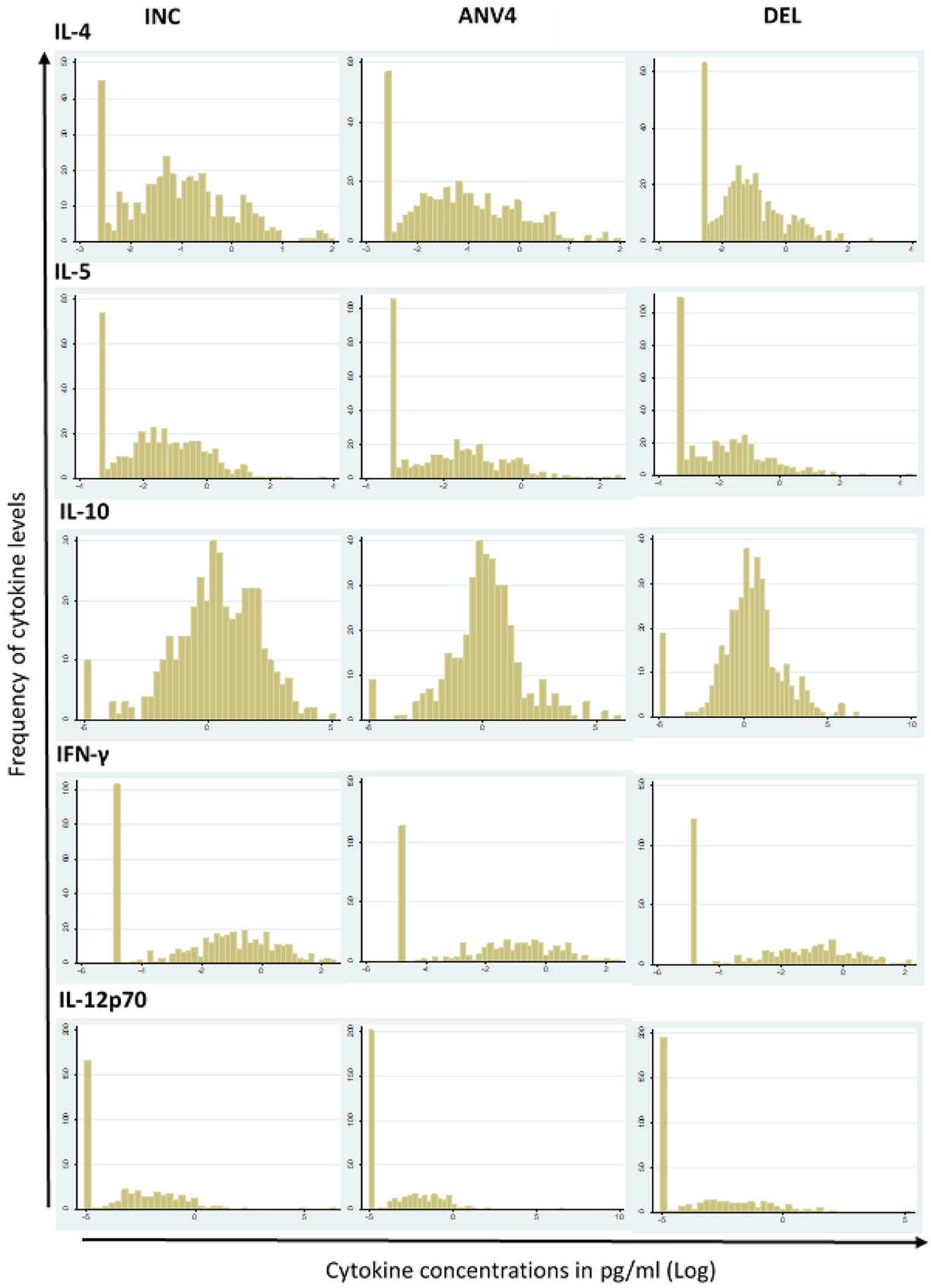

FIG 1 Overall distribution of cytokine according to peripheral plasma levels detected at inclusion, ANV4, and at delivery. The frequency of log-transformed levels of cytokine is represented.

TABLE 2 Cytokine concentrations during pregnancy[a]

Cytokine and time of assay during pregnancy	No. of peripheral plasma samples	Concn (pg/ml)			Threshold detection level (pg/ml)
		Median	*P* value	Range	
IL-4			0.075		0.072
INC	390	0.325		0.072–7.549	
ANV4	388	0.305		0.072–7.299	
DEL	387	0.274		0.072–15.950	
IL-5			<0.001		0.034
INC	390	0.212		0.034–41.444	
ANV4	388	0.152		0.034–12.727	
DEL	387	0.134		0.034–78.234	
IL-10			0.015		0.007
INC	390	1.473		0.007–177.155	
ANV4	388	1.171		0.007–435.557	
DEL	387	1.504		0.007–954.225	
IFN-γ			0.139		0.007
INC	390	0.233		0.007–11.740	
ANV4	388	0.197		0.007–12.218	
DEL	387	0.164		0.007–9.238	
IL-12p70			0.043		0.006
INC	390	0.039		0.006–621.541	
ANV4	388	0.006		0.006–756.269	
DEL	387	0.006		0.006–187.289	

[a]Cytokine median (range) concentrations during pregnancy at inclusion (INC), ANV4, and at delivery (DEL) were compared with Kruskal-Wallis test. *P* values of ≤0.05 were considered statistically significant.

infection with *P. falciparum* was increased ($P < 0.001$, OR = 2.65). Maternal age was independently associated with a decreased risk of infection (20 to 30 years, $P = 0.010$, OR = 0.47; >30 years, $P = 0.001$, OR = 0.31), while an increased number of ANV ($P = 0.003$, OR = 1.46) was also independently associated with a significantly increased risk of *P. falciparum* infection during pregnancy.

With respect to the number of episodes of *P. falciparum* infection during pregnancy, there was a positive association with IL-12p70 levels at inclusion ($P = 0.020$; incidence ratio [IR], 1.01). Of particular note, we did not find any relationship between IL-10 concentrations at inclusion and either the presence or the number of episodes of *P. falciparum* infection during pregnancy.

The same multivariate analysis, performed to analyze the association between anemia and cytokine concentrations, did not reveal any effect of one on the other (data not shown).

(iv) Cytokine levels predict pregnancy outcomes. The relationships between cytokine concentrations during pregnancy and PTB or SGA babies were analyzed after adjustment for *P. falciparum* infection and anemia (Table 7).

In the context of PTB, increasing concentrations of IL-12p70 at inclusion and of both IL-5 and IL-10 at ANV4 were associated with a higher risk of PTB ($P = 0.005$, OR = 9.42; $P = 0.004$, OR = 7.43; and $P = 0.001$, OR = 1.02, respectively). In contrast, increasing concentrations of IL-12p70 ($P = 0.010$, OR = 0.16) and IL-4 ($P = 0.009$, OR = 0.10) at ANV4 and, separately, of IL-5 at delivery ($P = 0.038$, OR = 0.11) were associated with a lower risk of PTB.

In the context of SGA, increasing IL-5 levels at inclusion displayed an association of borderline significance with a higher risk of SGA ($P = 0.054$, OR = 1.41), while at delivery the association was significant but reversed ($P = 0.049$, OR = 0.42).

Independently of the associations with cytokines, multigravid women had a significantly lower risk of SGA ($P = 0.022$, OR = 0.42), while women with more than three episodes of anemia had a significantly higher risk ($P = 0.041$, OR = 2.47).

TABLE 3 Cytokine concentrations in the groups of women[a]

Cytokine and time during pregnancy	Control (n = 60)		PAM (n = 170)			Anemia (n = 264)			PTB (n = 20)			SGA (n = 64)		
	Median	IQR	Median	IQR	P value[b]	Median	IQR	P value	Median	IQR	P value	Median	IQR	P value
IL-4														
INC	0.356	0.192–0.703	0.296	0.138–0.634	0.318	0.333	0.149–0.682	0.536	0.282	0.233–0.507	0.616	0.297	0.152–0.702	0.399
ANV4	0.333	0.188–0785	0.265	0.126–0.723	0.138	0.309	0.132–0.699	0.311	0.236	0.091–0.370	0.029	0.266	0.139–0.641	0.310
DEL	0.310	0.169–0.737	0.256	0.144–0.456	0.301	0.256	0.134–0.567	0.420	0.264	0.155–0.346	0.184	0.237	0.120–0.432	0.173
IL-5														
INC	0.260	0.066–0.562	0.244	0.082–0.722	0.458	0.202	0.082–0.596	0.847	0.276	0.150–0.744	0.337	0.181	0.051–0.722	0.815
ANV4	0.175	0.034–0.336	0.150	0.036–0.388	0.902	0.161	0.044–0.356	0.751	0.190	0.048–0.283	0.842	0.139	0.037–0.316	0.834
DEL	0.137	0.058–0.408	0.147	0.034–0.325	0.520	0.137	0.034–0.347	0.403	0.179	0.034–0.324	0.551	0.118	0.034–0.318	0.169
IL-10														
INC	0.746	0.245–1.554	2.837	0.788–8.342	<0.001	1.809	0.692–6.428	<0.001	3.905	0.917–6.572	0.003	2.679	0.594–6.288	<0.001
ANV4	0.728	0.199–1.336	1.907	0.924–3.659	<0.001	1.335	0.641–2.822	<0.001	1.960	0.509–5.875	0.008	1.275	0.692–3.357	<0.001
DEL	0.865	0.320–1.858	2.137	0.966–5.748	<0.001	1.526	0.608–3.821	0.007	3.402	1.641–5.159	0.002	2.089	0.951–7.262	<0.001
IFN-γ														
INC	0.280	0.007–0.869	0.246	0.007–0.787	0.708	0.221	0.007–0.821	0.657	0.286	0.025–0.851	0.887	0.255	0.023–0.869	0.830
ANV4	0.280	0.023–0.854	0.162	0.007–0.673	0.144	0.229	0.007–0.722	0.416	0.129	0.007–0.530	0.171	0.290	0.020–0.677	0.596
DEL	0.157	0.062–0.587	0.164	0.007–0.587	0.235	0.185	0.007–0.669	0.525	0.150	0.007–0.393	0.298	0.185	0.007–0.565	0.511
IL-12p70														
INC	0.056	0.006–0.224	0.028	0.006–0.181	0.390	0.036	0.006–0.219	0.558	0.027	0.006–0.185	0.681	0.037	0.006–0.197	0.527
ANV4	0.034	0.006–0.133	0.006	0.007–0.147	0.320	0.007	0.006–0.166	0.599	0.007	0.006–0.108	0.460	0.014	0.006–0.206	0.803
DEL	0.030	0.006–0.180	0.007	0.006–0.125	0.380	0.010	0.006–0.160	0.681	0.007	0.006–0.054	0.060	0.007	0.006–0.100	0.386

[a]The concentrations of each cytokine are represented by clinical groups (PAM, mothers who experienced *P. falciparum* infection during pregnancy; anemia, mothers who had anemia during pregnancy; PTB, women with preterm birth babies; SGA, women who delivered babies with low birth weight for gestational age). Each group was compared to a control group of mothers with no infection, no anemia, and with term-delivered babies and adequate birth weight for gestational age, using the Kruskal-Wallis test. IQR, interquartile range.

[b]P values of ≤0.0008 were considered statistically significant following application of Bonferroni correction.

TABLE 4 Cytokine levels as a function of the number of *P. falciparum* infections during pregnancy[a]

Cytokine and time	Cytokine level by no. of infections						
	None (n = 227)		One (n = 112)		Two or more (n = 58)		
	Median	IQR	Median	IQR	Median	IQR	*P* value
IL-4							
INC	0.359	0.192–0.664	0.304	0.169–0.634	0.291	0.114–0.622	0.370
ANV4	0.320	0.170–0.674	0.268	0.132–0.682	0.262	0.078–0.781	0.282
DEL	0.284	0.151–0.648	0.274	0.155–0.464	0.234	0.091–0.450	0.354
IL-5							
INC	0.194	0.059–0.512	0.257	0.001–0.766	0.211	0.082–0.652	0.098
ANV4	0.154	0.034–0.338	0.153	0.044–0.399	0.141	0.034–0.380	0.946
DEL	0.124	0.034–0.363	0.153	0.034–0.319	0.148	0.034–0.392	0.890
IL-10							
INC	1.192	0.352–3.029	2.597	0.725–7.528	3.468	0.945–10.328	<0.001
ANV4	0.813	0.365–1.626	1.716	0.751–3.489	2.555	1.098–9.065	<0.001
DEL	1.213	0.442–2.773	2.210	0.977–5.748	1.708	0.956–4.224	<0.001
IFN-γ							
INC	0.229	0.007–0.827	0.246	0.007–0.721	0.251	0.007–0.883	0.971
ANV4	0.229	0.007–0.714	0.153	0.007–0.734	0.173	0.007–0.590	0.377
DEL	0.154	0.007–0.683	0.196	0.007–0.659	0.048	0.007–0.328	0.058
IL-12p70							
INC	0.043	0.006–0.215	0.041	0.006–0.153	0.021	0.006–0.229	0.730
ANV4	0.025	0.006–0.133	0.006	0.006–0.149	0.006	0.006–0.146	0.317
DEL	0.015	0.006–0.162	0.010	0.006–0.134	0.006	0.006–0.092	0.370

[a]Comparison of cytokine levels by number of *P. falciparum* infections during pregnancy, with the exclusion of infections observed at delivery. *P* values are from comparisons between mothers who had no PAM, mothers who had only one infection during pregnancy, and mothers who had at least 2 episodes of PAM using the Kruskal-Wallis test. *P* values of ≤0.003 were regarded as statistically significant following application of Bonferroni correction.

DISCUSSION

The primary aim of the study described here was to determine whether the levels of circulating cytokines, measured at different moments during a longitudinal study of pregnancy-associated malaria in a cohort of Beninese, reliably reflected the different pregnancy outcomes recorded. Our basis for the study was an observation made in our

TABLE 5 Multivariate analysis of impact of *P. falciparum* infection on cytokine levels during pregnancy[a]

Cytokine and time	Variable and associated covariable	Occurrence of *P. falciparum* infection	No. of samples	Coef[b]	95% CI[c]	*P* value
Each time point						
IFN-γ						
INC	*P. falciparum* infection		70	0.43	0.38, 0.82	0.032
ANV4	*P. falciparum* infection		28	−0.06	−0.58, 0.45	0.805
DEL	*P. falciparum* infection		59	0.67	0.21, 1.12	0.004
IL-10						
INC	*P. falciparum* infection		70	11.19	8.19, 14.20	<0.001
	Gravidity (≥2)		333	−4.43	−7.46, −1.40	0.004
ANV4	*P. falciparum* infection		28	29.01	17.47, 40.56	<0.001
	Gravidity (≥2)		333	−11.33	−19.34, −3.32	0.006
DEL	*P. falciparum* infection		59	45.95	26.82, 65.08	<0.001
	Gravidity (≥2)		333	−4.15	−21.51, 13.20	0.638
Delivery						
IL-10		At least one episode of *P. falciparum* infection	397	24.01	1.52, 46.51	0.036
		No. of episodes of *P. falciparum* infection	397	−13.83	−25.20, −2.46	0.017

[a]At each time point, the independent effect of *P. falciparum* infection on cytokine levels was detected after adjusting for anemia, PTB, and SGA. A total of 187 women had at least 1 infection during pregnancy. Multivariate analysis was performed using all the factors that remained from the univariate analysis. Cytokine concentrations at delivery were adjusted on *P. falciparum* infection at delivery before testing their association with occurrence of *P. falciparum* infection during pregnancy. Only data from the multivariate regression model are shown. *P* values of ≤0.05 were regarded as statistically significant.

[b]Coef represents the average variation of cytokine concentrations associated with the presence of *P. falciparum* infection, number of *P. falciparum* infections, and other covariables. Gravidity was used as a categorical variable. Other factors included in the models as covariates are maternal age, gestational age, education level, IPT, ANV, birth weight, and use of bed nets.

[c]Represents 95% confidence intervals.

TABLE 6 Effect of cytokine levels at inclusion on *P. falciparum* infection during pregnancy[a]

Cytokine at inclusion and covariable associated	No. of samples	IR[b]	OR[c]	95% CI[d]	*P* value
Presence of infection					
IL-12p70	390		2.65	1.90, 3.69	<0.001
ANV no.	397		1.46	1.13, 1.88	0.003
IFN-γ	390		0.82	0.69, 0.99	0.035
ANV no.	397		1.32	1.04, 1.67	0.022
Maternal age	392				
20–30 yr	214		0.47	0.27, 0.84	0.010
>30 yr	90		0.31	0.16, 0.60	0.001
No. of infections					
IL-12p70	390	1.01		1.00, 1.02	0.020
ANV no.	397	1.17		1.03, 1.33	0.016
Birth wt	397	1.00		0.99, 1.01	0.018

[a]After adjusting for anemia, PTB, and SGA, cytokine levels were also adjusted on *P. falciparum* infection at inclusion to detect their independent effect on *P. falciparum* infection occurring during pregnancy.
[b]IR, incidence rate ratio. OR and IR represent the risk of *P. falciparum* infection and the risk of increasing incidence of *P. falciparum* infection during pregnancy, respectively. Maternal age was used as a categorical variable. Other factors included in the models as covariates are gravidity, gestational age, education, IPT, ANV, birth weight, and use of bed nets.
[c]OR, odds ratio.
[d]Represents 95% confidence intervals. Multivariate analysis was performed using all the factors that remained from the univariate analysis. Only data from the multivariate regression model with a *P* value of ≤0.05 are shown and regarded as statistically significant.

own earlier study of cytokines in placental plasma at delivery. In that study, we found that low levels of both IFN-γ and IL-5 were associated with the delivery of SGA babies and, thus, could represent markers of such a poor pregnancy outcome (25). Other studies, including our own, have also consistently shown that high levels of IL-10 present in placental plasma are closely associated with the occurrence of infections with *P. falciparum* during pregnancy.

Here, multivariate analyses were performed to determine (i) whether circulating cytokine concentrations at different time points of pregnancy were associated with either *P. falciparum* infections or poor pregnancy outcomes; (ii) the predictive value of circulating cytokine levels at inclusion with respect to *P. falciparum* infection and poor pregnancy outcomes; and (iii) the associations between episodes of *P. falciparum* infection and of anemia during pregnancy and the levels of cytokines at delivery.

Considering IL-10, multivariate analyses showed that high concentrations are strongly associated at each time point during pregnancy with *P. falciparum* infections. In addition, increased IL-10 levels observed at delivery were associated with *P. falciparum* infections that had occurred during pregnancy. Thus, these observations are consistent with the results of our own previous studies as well as those of others (25, 31–33). IL-10 is a key immunoregulatory molecule during infection. That IL-10 exerts direct effects on $CD4^+$ T cells, inhibiting proliferation and the production of IL-2, IFN-γ, IL-4, IL-5, and TNF-α, is well documented (34–36). Thus, IL-10 can suppress both Th1- and Th2-type responses. That capacity is consistent with our observations here of declining concentrations of both Th1- and Th2-type cytokines, significant in the case of both IL-5 and IL-12p70, in the face of sustained infection-induced IL-10 levels throughout pregnancy. Placental infection with *P. falciparum* is characterized by parasite-infected erythrocyte sequestration associated with often pronounced inflammation leading to detectable proinflammatory markers in peripheral blood (37). Thus, as a direct reflection of infection in the placenta, the inflammatory response and ensuing pathology can lead to poor pregnancy outcomes. We speculate that the strong IL-10 response observed in association with infection is a driving force in suppressing both potentially protective as well as pathological immune responses to maintain a healthy

TABLE 7 Multivariate analysis of cytokine levels and pregnancy outcomes[c]

Pregnancy outcome, cytokine or covariable, and time of cytokine assays	No. of samples	OR[a]	CI 95%[b]	*P* value
PTB				
IL-12p70				
INC	390	9.42	1.99, 44.47	0.005
ANV4	388	0.16	0.04, 0.64	0.010
DEL	387	0.16	0.00, 14.09	0.419
IL-4				
INC	390	1.19	0.16, 8.65	0.860
ANV4	388	0.10	0.02, 0.57	0.009
DEL	387	0.50	0.02, 14.02	0.687
IL-10				
INC	390	0.99	0.91, 1.07	0.789
ANV4	388	1.02	1.01, 1.03	0.001
DEL	387	0.99	0.98, 1.01	0.733
IL-5				
INC	390	0.46	0.11, 1.81	0.268
ANV4	388	7.44	1.91, 28.97	0.004
DEL	387	0.12	0.01, 0.89	0.038
SGA				
IL-5				
INC	390	1.41	0.99, 2.00	0.054
ANV4	388	0.59	0.13, 2.78	0.510
DEL	387	0.42	0.18, 0.99	0.049
Gravidity (≥2)	333	0.42	0.20, 0.88	0.022
No. of episodes of anemia	369			
≥1 and ≤3	47	1.53	0.39, 4.17	0.506
>3	217	2.47	1.03, 5.92	0.041

[a]OR, odds ratio. OR represents the risk of occurrence of PTB or SGA.
[b]Represents 95% confidence intervals.
[c]Cytokine concentrations were adjusted for the presence of anemia and *P. falciparum* infection to detect the independent association between cytokine levels and PTB on one hand and SGA on the other hand. All factors, along with gravidity, were included in univariate analysis. Factors with *P* values of <0.2 were included in multivariate analysis. Gravidity and number of episodes of anemia are used as categorical variables. Other factors included in the models as covariates are maternal age, gestational age, education, IPT, ANV, birth weight, and use of bed nets. Only cytokines remaining associated in the multivariate regression model are shown. *P* values of ≤0.05 were regarded as statistically significant.

pregnancy. Thus, parasite persistence in the placenta is an unintended consequence of maternal efforts to control inflammatory responses. Separately, we did notice lower levels of IL-10 at ANV4 compared with the levels found either at inclusion or at delivery, an observation that was consistent across all groups, regardless of their infection or other status (Tables 2 and 3). We speculate that this particular finding is related to women's changing overall immunological status during pregnancy that is thought to progress from an initial predominantly proinflammatory state to a more Th2-dominated noninflammatory environment before reverting to a proinflammatory state late in pregnancy prior to parturition (4). Thus, the comparatively lower levels of IL-10 seen at ANV4 would correspond to the noninflammatory period during which, logically, the necessity for IL-10 is reduced. Perhaps somewhat paradoxically, the circulating concentration of IL-10 at delivery also was found to be significantly lower in women who experienced multiple infections during pregnancy (Table 5, lower rows). This finding could reflect the fact that, upon detection, infections were always immediately treated, as stipulated in the study protocol. Therefore, in the case of those with multiple infections, it is plausible that proinflammatory responses, and the associated anti-inflammatory IL-10-led responses, will have had insufficient time to be well-established and, hence, to persist in the case of repeated treatments. This would be entirely consistent with our published findings concerning the association between submicroscopic infections and circulating cytokine levels (33).

We also observed increased IL-10 levels associated with *P. falciparum* infection in primigravid compared with multigravid women. In high-transmission areas, primigravidae are at greater risk of placental infection, whereas such a gravidity effect is less marked in low-transmission areas and absent from areas with epidemic malaria (38, 39). Gravidity-specific immunity, naturally acquired through consecutive pregnancies, contributes to resistance to placental infection with *P. falciparum*, reduces parasite densities, and prevents disease in areas of high and stable transmission. Thus, among primigravidae who lack specific acquired immunity, severe anemia and the delivery of underweight babies are more frequent. In addition, although the risk of maternal infection is reported to be highest during the second trimester (40), data from our own earlier studies suggest that *P. falciparum* infections can be particularly harmful at the beginning and at the end of pregnancy (30). This is consistent with our current observation concerning the association of *P. falciparum* infection with higher circulating levels of IL-10 in primigravid compared with multigravid women. Multivariate analysis of the predictive value of the cytokines assessed here did not reveal elevated IL-10 at inclusion to be associated with the risk of *P. falciparum* infection during pregnancy. In the STOPPAM study conducted in Tanzania, the combination of increased IL-10 and IP10 with decreased RANTES was predictive of infection with *P. falciparum* regardless of gestational age (31). Overall, the data suggest that IL-10 can be useful as a diagnostic rather than a prognostic marker of *P. falciparum* infection during pregnancy.

In the context of IL-12p70, increased levels of this proinflammatory cytokine at inclusion were associated with a significantly increased prospective risk both of *P. falciparum* infection during pregnancy as well as of preterm birth. On the other hand, increased levels at the inclusion of IFN-γ, a Th1-type proinflammatory cytokine, were associated with a significantly decreased prospective risk of *P. falciparum* infection during pregnancy, whereas when infections were present (at inclusion and at delivery), the levels of this cytokine were slightly but significantly elevated. These seemingly contrasting associations for two different proinflammatory cytokines are not easily resolvable. IL-12 is required for optimal IFN-γ production *in vivo* during immune responses, particularly during bacterial or parasitic infections (41). Circulating monocytes are the primary source of bioactive IL-12p70, in response to which NK cells readily produce IFN-γ, activating macrophages and enhancing their bactericidal activity and providing a mechanism of T-cell-independent macrophage activation during the early phases of innate resistance to infections (42). Separately, the ability of IL-12 to induce and maintain antigen-specific Th1-type responses is essential to control infections with many microbial pathogens (41, 43). We can only speculate that pregnancy-related and *P. falciparum* infection-related modulation of the production of different cytokines, including but not exclusive to Th1- and Th2-type responses, differentially affect their systemic levels depending on gestational age, for example, but also on the degree of chronicity of the infection. Successful pregnancy is characterized by a shift of the Th1/Th2 balance with a decrease of Th1-type cytokines and an increase in Th2-type cytokines. High circulating levels of IL-10 would be expected to inhibit monocyte activation and Th1-type cytokine synthesis, while low systemic levels of IL-12 in the face of elevated IL-10 levels are a feature of severe malaria in children (44, 45). Furthermore, downregulated IL-12p70, resulting from reduced transcription of its component IL-12p40, has been shown to be correlated with the uptake of *P. falciparum* hemozoin pigment and elevated levels of IL-10 in children with severe malarial anemia (46). Whether such mechanisms influence responses during the predominantly uncomplicated infections associated with pregnancy remains unknown.

We were particularly interested in the circulating cytokine profiles associated with the risk of preterm birth or small-for-gestational-age babies independently of *P. falciparum* infection and anemia. As already mentioned above, multivariate analysis revealed a significantly increased risk of preterm birth associated with increased levels of IL-12p70 at inclusion into the study, while the same association was also found for increased IL-5 and IL-10 levels at ANV4. Conversely, increased levels of IL-12p70 and IL-4

at ANV4 were found to be associated with a significantly reduced risk of preterm birth, as were increased levels of IL-5 at delivery. Many studies have reported that proinflammatory cytokines, such as IL-12, are implicated in the pathogenesis of poor pregnancy outcomes (29, 47, 48). However, most of those reports focused on peripheral or placental levels of cytokines at delivery. In the longitudinal study reported here, we know that between inclusion and ANV4, the women received 2 curative doses, spaced at least 1 month apart, of the antimalarial drug combination sulfadoxine-pyrimethamine. Thus, at least in those harboring *P. falciparum* infections when taking the treatment, it is plausible that the clearance of the parasites during that interval will have led to a change in the circulating cytokine profile, possibly explaining the contrasting associations observed at the 2 different time points during pregnancy.

We found that an increased risk of delivering an SGA baby was associated with increased levels of IL-5 at inclusion but that increased levels of the same cytokine at delivery were, conversely, associated with a significantly reduced risk of having a baby who was SGA, with the obverse clearly being that low levels of IL-5 were associated with an increase in such a risk. Thus, the latter finding is entirely consistent with the observation in our earlier study, namely, that low levels of IL-5 in placental plasma equated to an increased risk of having an SGA baby. In that study, we also observed the same association with increased risk of SGA babies for low levels of placental IFN-γ, but this was not the case for the levels of circulating IFN-γ assessed here. Nonetheless, it is striking that the circulating levels of the same cytokine, IL-5, measured at delivery appear here to distinguish two groups with distinctly different poor pregnancy outcomes, i.e., those with low circulating levels of IL-5 at delivery were at greater risk of either PTB or SGA. Whether the immunosuppressive activity of infection-induced IL-10 plays a determining role in this scenario and precisely how the suppression of a Th2-type cytokine might be mechanistically linked to either outcome of pregnancy are questions that remain open to speculation.

There are some limitations to our study that deserve mention. Although data from women found to be seropositive for HIV were excluded, the scope of the STOPPAM study did not allow for the detection of other coinfections, i.e., those of bacterial or helminth origin, for example, which could have influenced the cytokine measurements. In addition, we were unable to include an assessment of the possible influence of submicroscopic infections with *P. falciparum*, since we did not have relevant data for all women at all time points. It is also plausible that intermittent preventive treatment during pregnancy (IPTp) with sulfadoxine-pyrimethamine could, through the clearance of parasites, have modified cytokine concentrations. In the latter context, it is salient to note that, on average, the STOPPAM participants received their first and second doses of IPTp at 21 and 26 weeks of gestational age, respectively, placing the second dose approximately 8 weeks prior to the ANV4 sampling for the study here. We speculate that such an interval is sufficiently long to preclude any direct influence of the treatment on cytokine concentrations.

MATERIALS AND METHODS

The participants for this study were drawn from the EU-funded STOPPAM (Strategies to Prevent Pregnancy-Associated Malaria) study cohort. The STOPPAM study was conducted in Benin from 2008 to 2012 and involved the recruitment of 1,037 pregnant women who were monitored until delivery. One of the primary stated goals of STOPPAM was to quantify the pathophysiological and immunological effects of PAM. The study was approved by the ethics committees of the Faculty of Health Sciences of the University of Abomey-Calavi in Benin and of the Institute of Research for Development (IRD) in France.

Study area. Participants were enrolled in three health centers with maternity clinics (Comé, Akodeha, and Ouedeme Pedah), located in the district of Comé in Mono Province, southwestern Benin. Comé is predominantly urban, while the two other centers are in rural settings and are located 10 km apart. The area is considered moderate for malaria transmission, with two peaks during the rainy seasons (April to July and October to November) (49).

Study design and population. Women with a gestational age of fewer than 24 weeks who were resident for more than 6 months within 15 km of the health center and who were planning to deliver in the hospital were included after having given written informed consent (50). They were monitored at each scheduled monthly antenatal visit (ANV) for clinical and biological information. From the second trimester onwards, and according to the national guidelines at the time, they received two doses of

sulfadoxine-pyrimethamine (SP) as part of intermittent preventive treatment during pregnancy (IPTp) spaced at least 1 month apart.

The current study involved a subgroup of 400 women selected according to the pathological outcomes of pregnancy in order to quantify cytokine levels in peripheral plasma samples. In this context, 4 groups were defined: women who experienced *P. falciparum* infection during pregnancy, mothers who had anemia during pregnancy, women with preterm births (PTB), and women who delivered babies who were small for their gestational age (SGA).

P. falciparum infection during pregnancy was defined as a positive thick blood smear after microscopic examination of peripheral blood. Examination for *P. falciparum* infection was routinely conducted at each ANV and during emergency visits. All infected women were treated with quinine or SP per national guidelines.

Maternal anemia was assessed at each ANV and was defined as a hemoglobin level of $<$10 g/dl. PTB babies were those born at a gestational age of $<$37 weeks, as defined by ultrasound examination conducted before 24 weeks of GA. SGA babies were those with fetal growth restriction classified according to sex-specific charts for birth weights in less than the 10th percentile of fetal weight for gestational age (51). Samples from confirmed HIV-positive patients were not used in this study.

Sample collection. Venous blood samples were collected from the women at each ANV in vacutainers with citrate phosphate dextrose adenine anticoagulant. After centrifugation, plasma samples were separated, aliquoted, and stored at −80°C until further use.

Plasma cytokine quantification. Plasma samples collected at inclusion (INC), ANV4 (4 months after inclusion), and at delivery (DEL) were thawed and were analyzed for cytokine detection. Levels of IL-4, IL-5, IL-10, IL-12p70, and IFN-γ were assessed using Cytometric Bead Array (CBA) enhanced sensitivity technology (BD Biosciences, San Diego, CA, USA) according to the manufacturer's instructions. Data were acquired by flow cytometry (BD FACSCalibur) and analyzed using FCAP Array software (BD, Soft Flow Hungary). The lower limits of detection were 0.072 pg/ml, 0.034 pg/ml, 0.007 pg/ml, 0.006 pg/ml, and 0.007 pg/ml for IL-4, IL-5, IL-10, IL-12p70, and IFN-γ, respectively.

Statistical analysis. We considered three periods during the pregnancy follow-up: INC (enrolment in the study), ANV4, and DEL. We chose inclusion for the first period of pregnancy instead of trimester categorization, because few women were included in the study during the first trimester of their pregnancy.

The study population was stratified into subgroups according to clinical and other characteristics that comprised (i) infection with *P. falciparum*, (ii) maternal anemia, (iii) intrauterine growth restriction (assessed by SGA), and (iv) PTB.

Cytokine concentrations of the different groups were compared with a control group comprised of mothers who had no plasmodial infection, no anemia, and babies who (i) were delivered at term and (ii) had adequate birth weight for gestational age. In addition, cytokine concentrations were compared according to the number of *P. falciparum* infections that occurred during pregnancy. The nonparametric Kruskal-Wallis test was used for these comparisons. Bonferroni's correction was applied to correct for the high number of statistical tests. To identify independent associations between cytokine levels and either PAM or pregnancy outcomes, we performed multivariate analysis. Unsegregated data were used instead of segregated subgroups to determine the independent effect of PAM or each pregnancy outcome, adjusted on the basis of each of the others, on the cytokine concentrations. We then investigated the influence of PAM or anemia at a given time point on cytokine levels measured at the same time, and the association between the occurrence of PAM or anemia during pregnancy and the cytokine concentrations observed at delivery, using linear regression models. For the latter, cytokine concentrations at delivery were adjusted for the presence of *P. falciparum* infection at delivery. Subsequently, we investigated the influence of cytokine concentrations at inclusion on the occurrence of PAM or anemia during pregnancy using logistic and Poisson regression models. Here, cytokine levels were also adjusted for the presence of *P. falciparum* infection at inclusion. Finally, the relationships between PTB and SGA with cytokine levels measured during pregnancy and at delivery were determined using logistic regression models. All the tests were performed including at the first step with all the factors described. Covariates with *P* values of $<$0.2 were included in multivariate models as well as other factors of interest (*P. falciparum* infection, anemia, PTB, SGA, and cytokine levels at INC, ANV4, and DEL) for a stepwise analysis to find the best model. In the final multivariate analysis, the association between cytokine concentration at a given time point and *P. falciparum* infection or pregnancy outcome was validated if a *P* value of $\leq$0.05 was obtained. The occurrence of PAM or anemia is defined by the presence of PAM or anemia (at least one episode of PAM or anemia) and the number of episodes of *P. falciparum* infection or anemia during pregnancy. Other factors included in the models as covariates were gravidity, maternal age, gestational age, education level, IPT, number of ANV, birth weight, and use of bed nets. Some factors were used as categorical variables (number of anemia events, number of infections, maternal age, gestational age at inclusion, gravidity, number of IPTp, number of ANV, and birth weight). In all cases, we performed separate analyses for each time point. All statistical analysis was performed using Stata/IC version 13 (StataCorp LP, College Station, TX).

SUPPLEMENTAL MATERIAL

Supplemental material is available online only.

SUPPLEMENTAL FILE 1, PDF file, 0.1 MB.

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