Placental Hypoxia during Placental Malaria

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Background. Placental malaria causes fetal growth retardation (FGR), which has been linked epidemiologically to placental monocyte infiltrates. We investigated whether parasite or monocyte infiltrates were associated with placental hypoxia, as a potential mechanism underlying malarial FGR.

Methods. We studied the hypoxia markers hypoxia inducible factor (HIF)-1α, vascular endothelial growth factor (VEGF), placental growth factor, VEGF receptor 1 and its soluble form, and VEGF receptor 2. We used real-time polymerase chain reaction (in 59 women) to examine gene transcription, immunohistochemistry (in 30 women) to describe protein expression, and laser-capture microdissection (in 23 women) to examine syncytiotrophoblast-specific changes in gene expression. We compared gene and protein expression in relation to malaria infection, monocyte infiltrates, and birth weight.

Results. We could not associate any hallmark of placental malaria with a transcription, expression, or tissue-distribution profile characteristic of a response to hypoxia, but we found higher HIF-1α levels ($P = .0005$) and lower VEGF levels ($P = .0026$) in the syncytiotrophoblasts of cases of malaria than in those of asymptomatic control placentas.

Conclusions. Our data are inconsistent with a role for placental hypoxia in the pathogenesis of malaria-associated FGR. The laser-capture microdissection study was small, but its results suggest (1) that malaria affects syncytiotrophoblast-gene transcription and (2) novel potential mechanisms for placental malaria-associated FGR.

Placental malaria (PM) is characterized by the presence of malaria-infected erythrocytes in placental blood and is associated with both fetal and maternal mortality and morbidity [1, 2]. In particular, PM is a major preventable cause of low birth weight (LBW), and it is estimated that 200,000 children die as a result of PM-associated LBW every year [3]. In malaria-endemic areas, LBW is mainly due to fetal growth retardation (FGR) rather than to preterm delivery [1, 4], but the causes of this FGR remain unknown.

Placental hypoxia is an important cause of FGR, as has been shown by studies of high-altitude pregnancies (in which hypoxia is hypobaric in etiology [5]) or pre-eclampsia (in which placental insufficiency causes tissue hypoxia [6]). To date, there is no published study of the role that placental hypoxia plays in PM-associated FGR, despite clinical, pathological, and biological similarities between PM and pre-eclampsia (reviewed in [7]), and a long-proposed pathogenic role for placental hypoxia in PM-associated FGR. It has been suggested that some histopathological characteristics of PM, including thickening of basal membranes [8] and infiltration of monocytes into the intervillous spaces of the placenta (i.e., intervillositis) [9–11], could increase barriers to oxygen transport across the placenta [12]. It also has been postulated that the intervillous accumulation of inflammatory cells and infected erythrocytes could lead to placental and fetal hypoxia either by consumption of oxygen by the infiltrated cells or by decreasing the blood perfusion and effective surface area of

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maternal-fetal exchange [13, 14]; such placental dysfunctions could directly affect fetal development and lead to FGR [15, 16]. Increased syncytial knotting, which is considered to be a physiological adaptation to hypoxia [17], has been reported in malaria-infected placentas [18].

Hypoxia alters cellular gene-transcription profiles. A central hypoxia-responsive gene is the transcription factor hypoxia inducible factor 1 (HIF-1), a heterodimer of inducible HIF-1α and constitutive HIF-1β, which controls cellular response to hypoxia. Under normoxic conditions, HIF-1α protein is degraded by O_2-dependent ubiquitination and targeting to the proteasome [19–21]. In hypoxic tissues, HIF-1α protein is stabilized and can bind HIF-1β to form an active transcription factor that will alter the transcription of growth-factor genes involved in regulation of angiogenesis [22]. In particular, hypoxia is associated both with increased transcription of several molecules in placental tissue or cell lines—including vascular endothelial growth factor (VEGF) [23], Flt-1 (fems-like tyrosine kinase), or VEGFR1 [24], and its soluble form, sFlt-1 [25]—and with decreased transcription of placental growth factor (PlGF) [26]. Expression of the proteins VEGFR2 (or fetal liver kinase–1/kinase-insert domain–containing receptor [Flk-1/KDR]) [24, 27] and HIF-1α [28] is also up-regulated under hypoxic conditions in placental tissue.

To test the hypothesis that placental hypoxia plays a role in the pathogenesis of PM-associated FGR, we investigated whether PM infection, intervillositis, and LBW were associated with an expression profile characteristic of a response to hypoxia in normotensive pregnant women.

PARTICIPANTS, MATERIALS, AND METHODS

Recruitment of participants. The present study was approved by the College of Medicine Research Committee at the University of Malawi. From November 2001 until April 2005, placental tissue samples were collected from women who gave birth at Queen Elizabeth Central Hospital in Blantyre, Malawi, and who were participating in a study of interactions between HIV and PM [29]. For the present study, samples were selected from HIV-uninfected participants, on the basis of normal Apgar scores at 1 and 5 min and the absence of maternal complications (other than PM) such as hypertension, pre-eclampsia, chorioamnionitis, or maternal anemia. Placental histology was examined to classify eligible women into 1 of 4 groups: (1) those who previously had been infected with malaria (presence of malaria-pigment deposits but no parasites; these women were not included in the present study), (2) those uninfected with malaria, (3) those with malaria parasites but without pigment-containing monocytes, and (4) those with parasites with pigment-containing monocytes. A sample size of 60 was selected on the basis of a previous study [30] in which highly significant differences in placental chemokine mRNA were found between groups stratified by malaria and monocyte densities. Within groups, women with the highest parasite or monocyte densities were selected from those for whom samples were available.

Tissue sampling and handling. Placental tissue samples were taken near the center of the placenta at $\leqslant$20 min after delivery. One set of samples was snap-frozen in liquid nitrogen for RNA extraction, another set was embedded in optimal-cutting-temperature medium before being frozen at −80°C, for laser-capture microdissection, and a last set was fixed in buffered formalin, for immunohistochemical (IHC) analysis and malaria-infection grading.

Laboratory and pathology testing. Peripheral-blood malaria infection was assessed on thick blood films stained with Field's stain. Biopsy samples of placenta were formalin-fixed and examined, and the presence and density of malaria parasitemia and the presence of malaria pigment (hemozoin) were noted. In women with malaria, the density of intervillous-space monocyte infiltrates was recorded, as described elsewhere [9]. Biopsy samples were examined for the presence of chorioamnionitis, as described elsewhere [31]. Maternal hemoglobin concentration was determined by use of a HemoCue hemoglobinometer (HemoCue). LBW was defined as $<$2500 g.

Real-time reverse-transcriptase polymerase chain reaction (RT-PCR). Snap-frozen placental tissue was thawed in RNA*later*-ICE (Ambion), according to supplier's recommendations, before being homogenized in Trizol RNA extraction reagent (Invitrogen) by use of a tissue homogenizer (Polytron). RNA extraction was conducted according to the supplier's recommendations. The total cellular RNA obtained was resuspended in RNase-free water (Ambion) and was kept at −80°C until used.

RNA was reverse-transcribed by use of Superscript III enzyme mix (InVitrogen) in a 20-μL reaction volume, according to the supplier's recommendations. The cDNA was diluted 1:4 in DNase-free water and was kept at −20°C.

Primers were those either identified in the literature or designed by Primer Express software, and all were validated for chemical analysis using SYBR Green and an ABI 7900HT real-time PCR machine (all from Applied Biosystems). Data were analyzed as described elsewhere [32]. In brief, RNA standards were synthesized for each gene and were added to a pool of cellular RNA, to be used as external standards. Up to 3 housekeeping genes validated for their use in placental comparative-expression studies [33] were used as internal standards; they included the genes for (1) TATA box binding protein, (2) the succinate dehydrogenase complex, subunit A, and (3) the tyrosine 3-monooxygenase/tryptophan 5-monooxygenase activation protein, zeta polypeptide. For accuracy and reliability, target-gene signals were normalized to the geometric mean of the 3 housekeeping genes, as recommended by previous studies [33, 34].

Laser-capture microdissection. The syncytiotrophoblast (SCT) layer of uninfected placentas ($n = 6$) and *Plasmodium falciparum*–infected placentas with ($n = 10$) or without ($n = 7$) intervillositis was isolated by laser-capture microdissection. These cases were selected on the basis of availability of additional placental samples, from within the group of 59 women studied at the whole-tissue level. All available samples were used. Tissue cryosections 5–7-μm thick were immobilized on SuperFrost PLUS slides (Fisher) and were air-dried for 10 min before being fixed by treatment with acetone for 10 min. After rehydration in diethyl pyrocarbonate–treated water, sections were lightly stained with methyl green (Sigma-Aldrich) and then were dehydrated in ethanol and cleared in xylene. Slides were kept in a desiccator until capture. Capture was performed by laser microdissection and pressure catapulting using a MicroBeam microscope (P.A.L.M. Microlaser Technologies). Captured material was catapulted directly into RNA extraction buffer (RLT buffer with β-mercaptoethanol; Qiagen), and RNA was extracted by use of an RNeasy Micro Kit (Qiagen), according to the supplier's recommendations. Purified RNA was eluted in RNase-free water (Ambion) and was kept at −80°C until used.

IHC analysis. IHC analysis was performed on 5-μm-thick paraffin-embedded tissue sections immobilized on silanized slides. Depending on the target, epitope retrieval was achieved by use of either 10 mmol/L citrate buffer, for KDR and VEGF, or Target Retrieval Solution (Dako), for PlGF, HIF-1α, and Flt-1. After the endogenous peroxidase was quenched by a 20-min incubation in 0.5% H_2O_2 in water, sections were incubated with primary antibodies for 1 h. Mouse monoclonal anti–HIF-1α (clone ESEE122 at 1:8000; Novus Biologicals), rabbit polyclonal anti-PlGF (CPP500 at 1:50; Cell Sciences), rabbit polyclonal anti-VEGF (sc-152 at 1:200), mouse monoclonal anti-KDR (sc-6251 at 1:100), or rabbit polyclonal anti–Flt-1 (sc-316 at 1/500), all from Santa Cruz were diluted in antibody diluent (S0809; Dako). After the sections were washed in PBS, 1 of the following biotinylated secondary antibodies, all from Dako, was added for 30 min: polyclonal rabbit anti-mouse (E0354 at 1:300, for KDR); polyclonal swine anti-goat, -mouse, or -rabbit (E0453 at 1:300, for VEGF and PlGF); or polyclonal goat anti-rabbit (E0432 at 1:300 for Flt-1). Biotinylated secondary (E0354 at 1:600) and tertiary (polyclonal swine anti-goat, -mouse, and -rabbit; E0453 at 1:200) antibodies were used for detection of HIF-1α.

For different cell types, intensity and consistency of staining were scored by 2 independent microscopists (P.B. and C.R.) blinded to the clinical presentation and were averaged on 20 microscopic fields. Intensity scores ranged from 0 (no staining) to 3 (very intense staining), and consistency scores ranged from 0 (no cell stained) to 3 (more than two-thirds the cells stained) [35].

Statistical tests. The Mann-Whitney test was used for numerical-value comparisons of 2 groups, and the Kruskal-Wallis test was used for numerical-value comparisons of 3 groups. The Mann-Whitney test was used for intergroup comparison of IHC scores (detailed above). All analyses were performed by Stata software (version 8.0). $P < .05$ was considered to be significant.

RESULTS

Participants' characteristics. The characteristics of the participants whose samples were used in the RT-PCR analysis are summarized in the top half of table 1. A total of 35 women with active PM and 24 uninfected control subjects were included in the study. Of the 35 women with PM, 22 (63%) had intervillositis, and 8 (23%) had LBW babies; none of the uninfected women had intervillositis, and only 1 of them delivered an LBW baby (figure 1*A*). Statistical differences in gestational age were ignored, because all deliveries were within the normal range for term deliveries.

When malaria cases were separated on the basis of the presence or absence of intervillositis, babies delivered by uninfected women had birth weights similar to those of babies delivered by infected women without intervillositis ($P = .86$), but the birth weights of babies delivered by patients with intervillositis were significantly lower than those of babies delivered either by infected women without intervillositis ($P = .027$) or by uninfected women ($P = .007$).

Transcription profiles are not characteristic of a response to hypoxia. Quantitative real-time RT-PCR was performed to look for an increase in transcription of HIF-1α, VEGF, Flt-1, and sFlt-1 and for a decrease in transcription of PlGF in malaria-infected samples, which would be a signature of tissue hypoxia. First, the quality of the extracted RNA was assessed by automated electrophoresis on RNA *Lab*Chip (Bio-Rad); all the samples included in the study had an rRNA 28s:18s ratio >1 (data not shown).

For none of the markers were transcript levels different between control subjects and malaria cases or between malaria cases with intervillositis and malaria cases without intervillositis (figure 2*A*). Samples were then classified according to other clinical or biological features of PM: intervillositis (regardless of malaria status [data not shown]) and LBW (figure 2*B*). Again, the transcription profiles of the groups were not different, for any classification criteria. These results suggest that none of the clinical or biological features of PM is specifically associated with placental hypoxia.

Laser-capture microdissection reveals differences in transcription profiles. Because monocytes can produce some of the markers quantified [36, 37], the presence of intervillositis in only a portion of the samples is a potential bias when groups are

Table 1. Participants' characteristics, as determined by reverse-transcriptase polymerase chain reaction (RT-PCR) and immunohistochemistry.

		Participants with malaria		Participants' birth weight	
	Control group	Without monocytes	With monocytes	Normal	Low[a]
RT-PCR	$n = 24$	$n = 14$	$n = 21$	$n = 52$	$n = 7$
Age, years	22 (20–25.5)	22.5 (21–28)	21 (20–24)	22 (20–26)	20 (18–21)
Parity	1.5 (1–3)	2 (1–4)	2 (1–3)	2 (1–3)	1 (1–2)
Maternal hemoglobin, g/dL	11.5 (10.5–12.45)	11.45 (10.4–12.5)	11.2 (10.4–12)	11.25 (10.4–12.4)	11.3 (10.5–12.4)
Gestational age, weeks	40 (39–41)	39 (39–40)	39 (38.5–40)	40 (39–40)	39 (38–39)
Maternal weight, kg	62.5 (56–66.5)	58.5 (55–63)	58 (55–62)	60.5 (55.25–64.75)	58 (55–61)
Maternal height, cm	158 (153–159)	157.5 (155–161)	154.5 (152–157)	156.5 (153.5–159)	154 (152–161)
Birth weight, g	3100 (2825–3400)	3135 (3000–3400)	2925 (2550–3150)	3100 (2900–3300)	2500 (2400–2500)
Immunohistochemistry	$n = 10$	$n = 10$	$n = 10$	$n = 28$	$n = 2$
Age, years	23 (22–26)	23 (22–26)	25 (21–28)	23 (22–28)	21 (21–23)
Parity	2 (2–3)	2 (1–3)	2.5 (2–4)	2 (1–3)	2 (2–2)
Maternal hemoglobin, g/dL	10.5 (9.4–11.4)	12.5 (10.6–12.7)	11.1 (10–11.2)	11.2 (10.1–12.5)	10.7 (10.5–10.7)
Gestational age, weeks	41 (40–42)	40 (39–40)	40 (39–41)	40 (40–41)	39 (35–39)
Maternal weight, kg	60 (56–73)	56 (51–60)	62 (57–64.5)	59 (53–65)	61 (56–61)
Maternal height, cm	153 (153–159)	155 (151–157)	155 (149.5–160)	155 (151–159)	161 (153–161)
Birth weight, g	3100 (3000–3400)	3100 (2700–3590)	2975 (2750–3300)	3100 (2950–3590)	2500 (2000–2500)

NOTE. Data are median (interquartile range) values. In the real-time RT-PCR study, gestational age differed regardless of the classification criteria used ($P \leq .05$) but stayed within the normal range for full-term deliveries; this difference was therefore ignored. Women who delivered low-birth-weight babies were of lower gravidity ($P = .026$) and were younger ($P = .025$) than those who delivered normal-birth-weight babies. The groups of participants in the immunohistochemistry analysis were comparable for all criteria (except for birth weight for the low-birth-weight group: $P = .019$).

[a] <2500 g.

compared. Using laser-capture microdissection, we specifically addressed the transcription profile of the SCT layer (figure 3*A*).

When control subjects were compared with malaria cases (regardless of the presence or absence of intervillositis), the only statistical differences noted were lower transcript levels of VEGF ($P = .0026$), and higher transcript levels of HIF-1α ($P = .0005$), in the SCT of infected placentas. Differences between the levels of VEGF in the SCT of malaria cases with intervillositis and those in malaria cases without intervillositis did not reach significance ($P = .49$) (figure 3*B*). Levels of HIF-1α in the SCT of malaria cases with intervillositis were similar to those of malaria cases without intervillositis ($P = .70$), but the levels in both of these groups were higher than those in control placentas ($P = .0001$ and $P = .0027$, respectively). When samples were classified on the basis of the presence or absence of intervillositis (regardless of malaria status [data not shown]) or on the basis of birth weight (figure 3*C*), there were no statistical differences. Taken together, these data suggest that the transcription profile of the SCT is not typical of a response to hypoxia, although they do indicate differences that were not detected when placental tissue was considered as a whole. Moreover, these differences appear to be associated with the presence of malaria infection, and not specifically with intervillositis, because they were present regardless of whether monocyte infiltrates were detected.

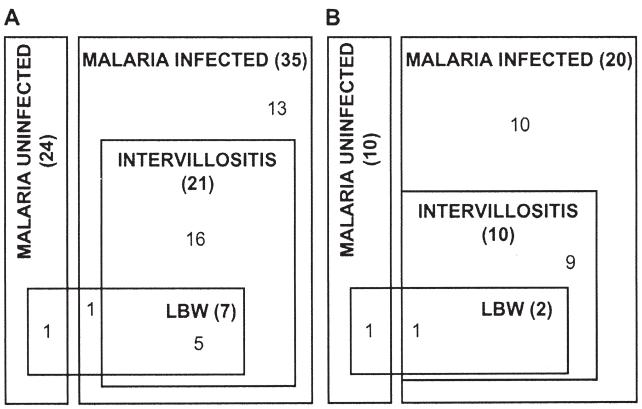

Figure 1. Clinical characteristics of the participants. As shown in panel *A,* 7 of the participants recruited for the assays using reverse-transcriptase polymerase chain reaction had a low birth weight (LBW): 5 of them had malaria with intervillositis, 1 had malaria without intervillositis, and 1 was a control subject. As shown in panel *B,* only 2 of the participants recruited for immunohistochemical analysis suffered from LBW: 1 was a control subject, and 1 had malaria. These 2 participants had similar immunohistochemical profiles, even when LBW was defined as small for gestational age ($n = 7$). Numbers denote the number of samples from each overlapping group.

Protein expression and tissue distribution is not characteristic of a response to hypoxia. To further validate these findings and look for a potential difference in the various hypoxia markers' protein expression and tissue distribution, we used IHC analysis to determine the protein-expression profiles of the different samples. Characteristics of the patients included in the

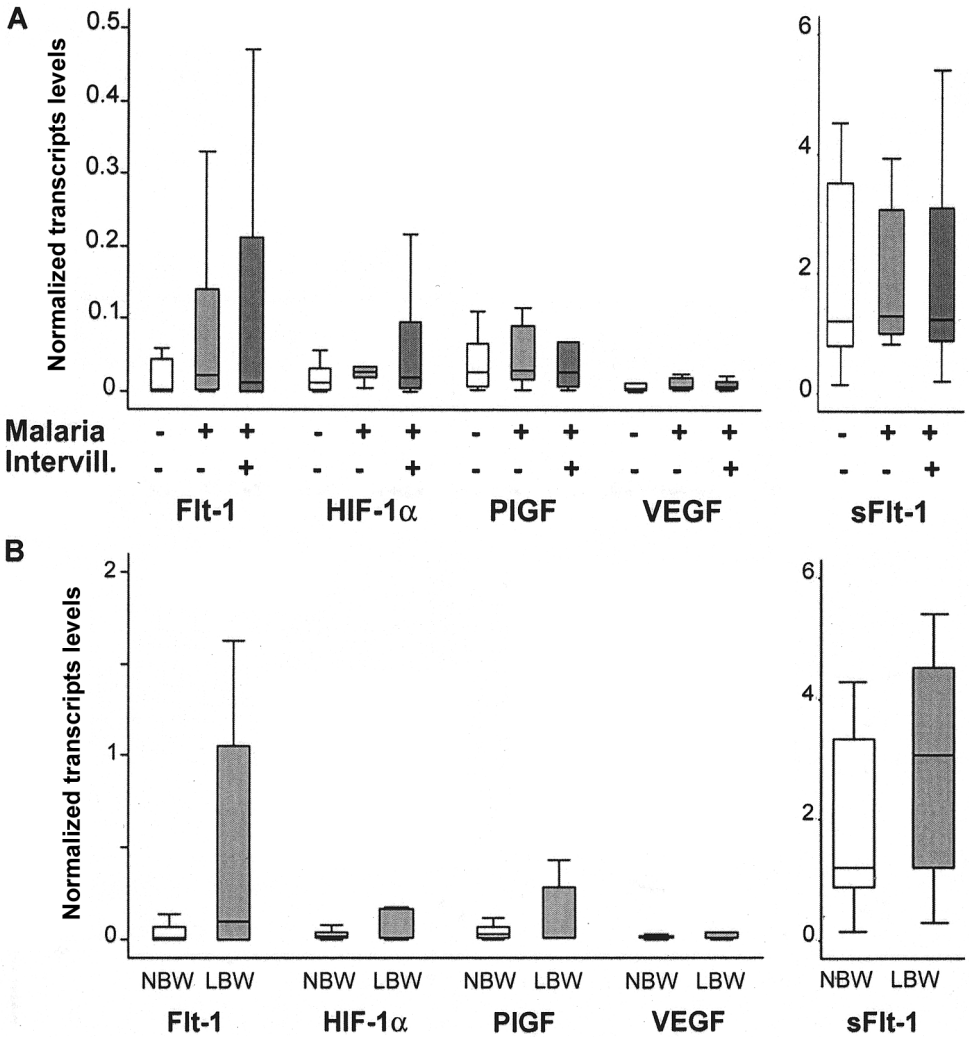

Figure 2. Transcription profiles for different clinical groups. The transcript levels of the different hypoxia markers were quantified in all participants, by absolute quantitative real-time reverse-transcriptase polymerase chain reaction, and were normalized to the expression of 3 placental housekeeping genes. As shown in panel *A,* transcription profiles were similar for 24 control subjects *(white bars),* 14 cases of malaria without intervillositis *(light-grey bars),* and 21 cases of malaria with intervillositis *(dark-grey bars).* As shown in panel *B,* there was virtually no difference between the transcription profiles for 52 participants with normal birth weight (NBW *[white bars]*) and those for 7 patients with low birth weight (LBW *[grey bars]*); $P > .05$, by Mann-Whitney test. In both panels, the whiskers extending from each bar denote the 5th and 95th percentiles, the top and bottom of each bar denote the interquartile values, and the horizontal line transecting each bar denotes the median value. Flt-1, vascular endothelial growth factor receptor–1; HIF, hypoxia-inducible factor; Intervill., intervillositis; PlGF, placental growth factor; sFlt-1, soluble Flt-1; VEGF, vascular endothelial growth factor.

IHC experiments are summarized in the bottom half of table 1. Only 2 patients delivered LBW babies (figure 1*B*). All groups are comparable for every criterion (except for those criteria—e.g., birth weight—that were used for classification), regardless of the classification used ($P \geqslant .057$).

For each placental tissue sample, we determined the expression and tissue distribution of VEGF, Flt-1, KDR, PlGF, and HIF-1α (figure 4). Both microscopists found the same expression and distribution profiles for each marker (data not shown); results are provided in table 2.

When samples were classified as being from control subjects or from malaria cases, no difference in consistency or intensity of staining was found, for any of the markers in any cell type. No difference was found when samples from patients with malaria were classified according to the presence or absence of intervillositis or when they were classified according to birth weight. However, when samples from all participants were classified according to the presence or absence of intervillositis, a more intense ($P = .037$) and consistent ($P = .041$) VEGF staining in the villous stroma was found in the samples with intervillositis, regardless of malaria status; however, these differences were not statistically significant after Bonferroni adjustment ($P > .01$). For no other marker did the staining patterns differ between these 2 groups. Taken together, the transcription and expression profiles suggest that neither PM nor LBW nor intervillositis is associated with placental hypoxia.

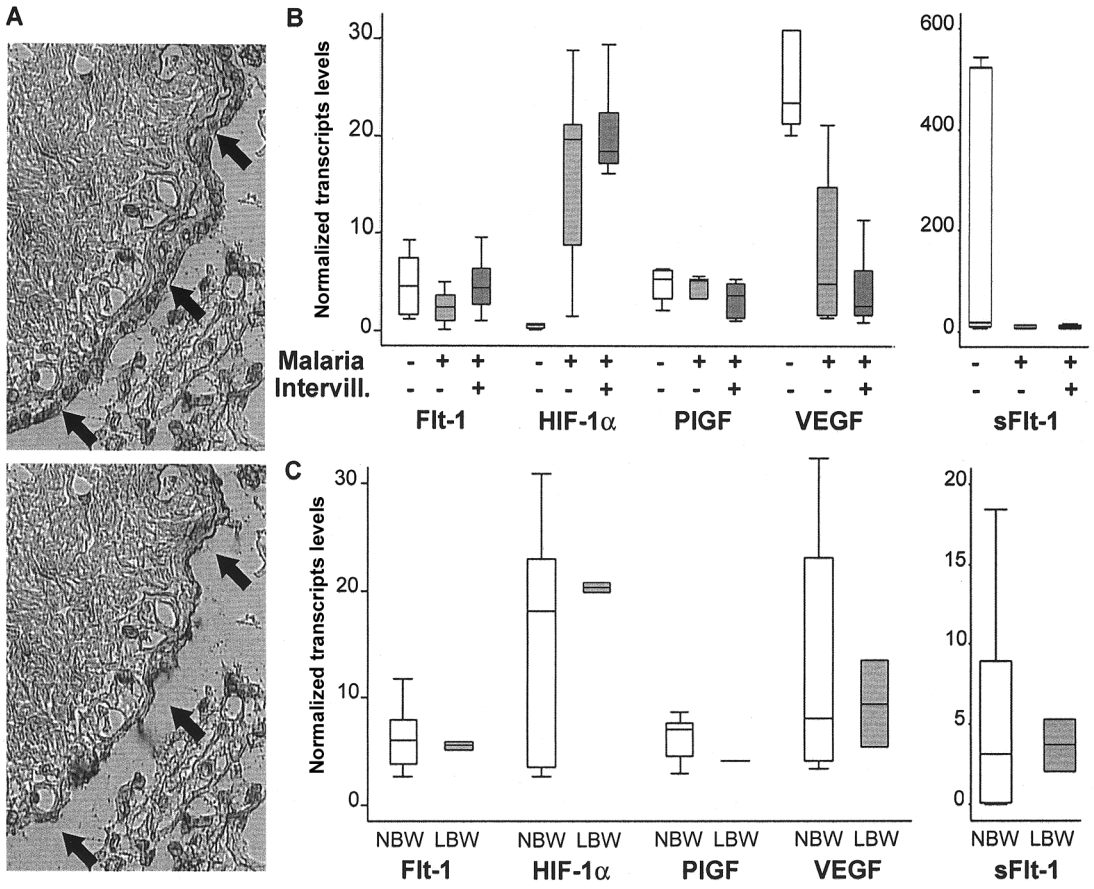

Figure 3. SCT-specific transcription profile. *A,* Syncytiotrophoblast specifically captured from cryosections of placental tissue by laser-capture microdissection. The transcription profiles (*B* and *C*) were determined by quantitative real-time reverse-transcriptase polymerase chain reaction and were normalized to the expression of a placental housekeeping gene. *B,* Comparison of transcription profiles for 6 control subjects *(white bars),* 7 cases of malaria without intervillositis *(light-grey bars),* and 10 cases with monocyte infiltrates *(dark-grey bars). C,* Similarity between transcription profiles for 21 participants with normal birth weight (NBW *[white bars]*) and those for 2 participants with low birth weight (LBW *[grey bars]*); $P > .05$, by Mann-Whitney test. In panels *B* and *C,* the whiskers extending from each bar denote the 5th and 95th percentiles, the top and bottom of each bar denote the interquartile values, and the horizontal line transecting each bar denotes the median value. Flt-1, vascular endothelial growth factor receptor–1; HIF, hypoxia-inducible factor; Intervill., intervillositis; PIGF, placental growth factor; sFlt-1, soluble Flt-1; VEGF, vascular endothelial growth factor.

DISCUSSION

It has been long suggested that placental hypoxia could play a role in PM-associated FGR, but direct evidence for such a role has been lacking. In the present study, we tested this hypothesis by looking for molecular evidence of placental hypoxia in PM.

Cellular response to hypoxia is orchestrated by the transcription factor HIF-1, which modifies the expression of several genes—in particular, those for angiogenic factors. Using a very reliable and sensitive quantitative real-time RT-PCR approach, we did not find, when we considered placental tissue as a whole, any association between any clinical or biological features of PM and a transcription profile characteristic of a molecular response to hypoxia.

The absence of transcriptional evidence for placental hypoxia in PM was further substantiated by the lack of association between the clinical and biological features of PM and expression patterns of hypoxia-specific markers as evidenced by IHC analysis. The staining patterns obtained were similar to those described elsewhere [27, 28, 38, 39]. When we used a previously validated scoring process [35] to quantify the expression of these markers, we found no evidence for a hypoxia-specific expression pattern in any of the groups. Because only 2 samples were classified as being from LBW cases, we also used an alternative definition of LBW—specifically, a birth weight that was below the 5th percentile of a normal fetal growth curve [40]—to compare normal-size ($n = 32$) and small-for-gestational-age ($n = 7$) infants; the 2 groups of infants had similar IHC profiles (data not shown). Taken together, these results strongly argue that placental hypoxia does not play a role in PM pathogenesis.

In contrast to a recent study that showed that PM is associated with increased sFlt-1 mRNA levels in normotensive primigravidas and in malaria-infected placentas with intervillositis, compared with control placentas [41], we did not find any difference

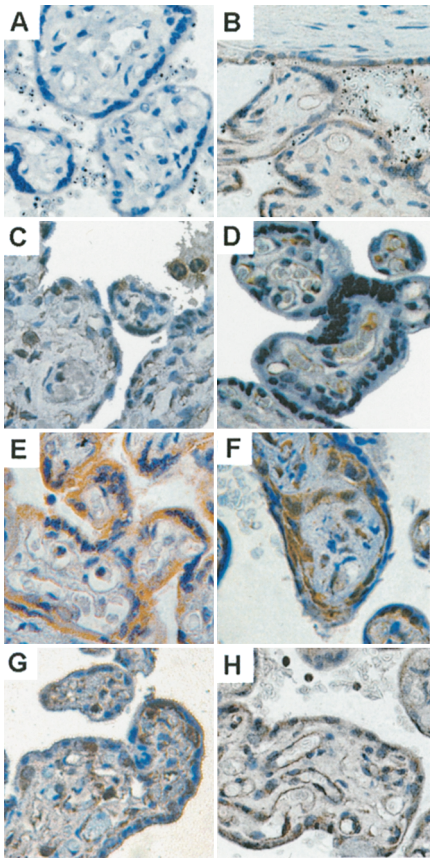

Figure 4. Examples of immunohistochemical staining. The expression and tissue distribution of the different hypoxia markers were assessed on fixed placental-tissue sections. *A,* Specificity of staining, as checked by omission of the primary antibody. *B,* Staining of placental growth factor in syncytium and endothelium of a malaria-infected placenta. *C,* Staining of cells positive for vascular endothelial growth factor receptor–1, or Flt-1, found in syncytium, villus-vessel walls, and Hofbauer cells. *D,* Staining of kinase insert domain-containing receptor, mainly in villus-vessel endothelium. In control placentas *(E),* staining of vascular endothelial growth factor was seen mainly in syncytium, but malaria-infected placentas with intervillositis *(F)* showed significantly more frequent and intense staining of stromal cells. However, staining of hypoxia-inducible factor–1α in uninfected placentas (*G*) was not different from that in malaria-infected placentas *(H)*—both showed staining of syncytium and stromal cells (possibly Hofbauer cells). Original magnification, 200x; color balance, luminosity, and contrast have been optimized.

in sFlt-1 RNA levels between the different groups when we considered either placental tissue as a whole or the SCT only. Differences in the recruitment criteria (we focused on normotensive women and included all gravidities, rather than just primigravidas) and in the technical approaches used (the Flt-1 antibody that we used recognizes only the membrane-bound form, unlike those which other studies used, which bind both the membrane-bound and the soluble forms) could explain these conflicting results. Moreover, each study was relatively small (~60 women) and suffers from some limitations in power. Further investigation of the interactions between malaria and hypertension and pre-eclampsia in first pregnancies may be warranted.

Because all the samples in the cohort that we studied presented similar transcription and expression profiles, it is possible that they could all be hypoxic as a consequence of labor or tissue handling. However, because we included only placentas from children with a normal Apgar score, labor-induced acute hypoxia is unlikely. Moreover, because transcript levels of HIF-1α have been shown to change very rapidly after the onset of cellular hypoxia, if all samples had suffered from hypoxia as a consequence of labor or tissue handling, then they all would have expressed similar levels of HIF-1α transcript. However, we did find a difference between the transcript levels of HIF-1α in the SCT of malaria-infected placentas and those in control placentas, which further rules out the possibility of a general induced hypoxia.

The increased transcript levels of HIF-1α in the SCT of malaria-infected placentas could reflect local inflammation [42]. Indeed, hypoxia-induced HIF-1α up-regulation is thought to occur at the level of protein stability (post-transcriptional). Thus, higher transcript levels of HIF-1α in the syncytium of malaria-infected placentas are likely to be a consequence of local inflammation that could be both the underlying cause of the molecular and protein changes noted and the driving force behind malaria-associated LBW. Because the transcript levels of HIF-1α in malaria cases were similar regardless of the presence and absence of monocyte infiltrates, which are markers of chronic inflammation, the increase in the transcript levels of HIF-1α might be due to either a more acute inflammation or the presence of infected erythrocytes. Testing of this hypothesis requires further study.

We did not find any association between the biological or clinical features of PM and a transcription or expression profile characteristic of a response to hypoxia. This finding argues against a role for placental hypoxia in PM-associated FGR pathogenesis. Although the role that placental hypoxia plays in the pathogenesis of FGR has been well described, particularly in animal models [43], it is not universally implicated in FGR. For example, several studies have found no difference between the expression of VEGF in normal placentas and that in FGR-affected placentas [44–46].

The present study does not rule out the possibility of fetal hypoxia, which could decrease fetal growth. Kingdom and Kaufmann have described a situation leading to fetal hypoxia in the absence of placental hypoxia when the fetoplacental perfusion is inadequate; this condition is known as "post-placental hypoxia" [47]. However, one study did not find ev-

Table 2. Immunohistochemistry scoring.

The table is available in its entirety in the online edition of the *Journal of Infectious Diseases.*

idence of FGR-associated fetal hypoxia [47]. Thus, longitudinal assessment of placental and fetal blood flow [48], combined with analysis of arterial gas in cord blood throughout pregnancy, appears to be essential to allow us to adequately address the impact that a potential fetal hypoxia has on intrauterine growth during PM.

Acknowledgments

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References

1. Desai M, ter Kuile F, Nosten F, et al. The global burden of malaria in pregnancy: what's known and where are the gaps? Lancet Infect Dis **2007**; 7:93–104.
2. Beeson JG, Duffy PE. The immunology and pathogenesis of malaria during pregnancy. Curr Top Microbiol Immunol **2005**; 297:187–227.
3. Steketee RW, Nahlen BL, Parise ME, Menendez C. The burden of malaria in pregnancy in malaria-endemic areas. Am J Trop Med Hyg **2001**; 64:28–35.
4. Guyatt HL, Snow RW. Impact of malaria during pregnancy on low birth weight in sub-Saharan Africa. Clin Microbiol Rev **2004**; 17:760.
5. Zamudio S. The placenta at high altitude. High Alt Med Biol **2003**; 4: 171–91.
6. Soleymanlou N, Jurisica I, Nevo O, et al. Molecular evidence of placental hypoxia in preeclampsia. J Clin Endocrinol Metab **2005**; 90:4299–308.
7. Brabin BJ, Johnson PM. Placental malaria and pre-eclampsia through the looking glass backwards? J Reprod Immunol **2005**; 65:1–15.
8. Ismail MR, Ordi J, Menendez C, et al. Placental pathology in malaria: a histological, immunohistochemical, and quantitative study. Hum Pathol **2000**; 31:85–93.
9. Rogerson SJ, Pollina E, Getachew A, Tadesse E, Lema VM, Molyneux ME. Placental monocyte infiltrates in response to *Plasmodium falciparum* infection and their association with adverse pregnancy outcomes. Am J Trop Med Hyg **2003**; 68:115–9.
10. Galbraith RM, Faulk WP, Galbraith GM, Holbrook TW, Bray RS. The human materno-foetal relationship in malaria. I. Identification of pigment and parasites in the placenta. Trans R Soc Trop Med Hyg **1980**; 74: 52–60.
11. Ordi J, Ismail MR, Ventura PJ, et al. Massive chronic intervillositis of the placenta associated with malarial infection. Am J Surg Pathol **1998**; 22: 1006–11.
12. Duffy PE. Immunity to malaria during pregnancy: different host, different parasite. In: Duffy PE, Fried M, eds. Malaria in pregnancy: deadly parasite, susceptible host. Washington, DC: Taylor & Francis, **2001**:71–126.
13. Kaushik A, Sharma VKS, Kumar R. Malaria placental infection and low birth weight babies. J Commun Dis **1992**; 24:65–9.
14. Steketee RW, Wirima JJ, Hightower AW, Slutzker L, Heymann DL, Breman JG. The effect of malaria and malaria prevention in pregnancy on offspring birthweight, prematurity, and intrauterine growth retardation in rural Malawi. Am J Trop Med Hyg **1996**; 55:33–41.
15. Garnham PCC. The placenta in malaria with special reference to reticulo-endothelial immunity. Trans R Soc Trop Med Hyg **1938**; 32: 13–35.
16. Watkinson M, Rushton DI. Plasmodial pigmentation of placenta and outcome of pregnancy in West African mothers. Br Med J (Clin Res Ed) **1983**; 287:251–4.
17. Heazell AEP, Moll SJ, Jones CJP, Baker PN, Crocker IP. Formation of syncytial knots is increased by hyperoxia, hypoxia and reactive oxygen species. Placenta **2007**; 28(Suppl A):S33–40.
18. Crocker IP, Tanner OM, Myers JE, Bulmer JN, Walraven G, Baker PN. Syncytiotrophoblast degradation and the pathophysiology of the malaria-infected placenta. Placenta **2004**; 25:273.
19. Salceda S, Caro J. Hypoxia-inducible factor 1alpha (HIF-1alpha) protein is rapidly degraded by the ubiquitin-proteasome system under normoxic conditions: its stabilization by hypoxia depends on redox-induced changes. J Biol Chem **1997**; 272:22642.
20. Huang LE, Gu J, Schau M, Bunn HF. Regulation of hypoxia-inducible factor 1alpha is mediated by an O2-dependent degradation domain via the ubiquitin-proteasome pathway. Proc Natl Acad Sci USA **1998**; 95: 7987.
21. Kallio PJ, Wilson WJ, O'Brien S, Makino Y, Poellinger L. Regulation of the hypoxia-inducible transcription factor 1alpha by the ubiquitin-proteasome pathway. J Biol Chem **1999**; 274:6519.
22. Reynolds LP, Redmer DA. Angiogenesis in the placenta. Biol Reprod **2001**; 64:1033–40.
23. Taylor CM, Stevens H, Anthony FW, Wheeler T. Influence of hypoxia on vascular endothelial growth factor and chorionic gonadotrophin production in the trophoblast-derived cell lines: JEG, JAr and BeWo. Placenta **1997**; 18:451.
24. Trollmann R, Amann K, Schoof E, et al. Hypoxia activates the human placental vascular endothelial growth factor system in vitro and in vivo: up-regulation of vascular endothelial growth factor in clinically relevant hypoxic ischemia in birth asphyxia. Am J Obstet Gynecol **2003**; 188: 517–23.
25. Li H, Gu B, Zhang Y, Lewis DF, Wang Y. Hypoxia-induced increase in soluble Flt-1 production correlates with enhanced oxidative stress in trophoblast cells from the human placenta. Placenta **2005**; 26:210.
26. Ahmed A, Dunk C, Ahmad S, Khaliq A. Regulation of placental vascular endothelial growth factor (VEGF) and placenta growth factor (PIGF) and soluble Flt-1 by oxygen--a review. Placenta **2000**; 21(Suppl A):S16–24.
27. Kumazaki K, Nakayama M, Suehara N, Wada Y. Expression of vascular endothelial growth factor, placental growth factor, and their receptors Flt-1 and KDR in human placenta under pathologic conditions. Hum Pathol **2002**; 33:1069–77.
28. Rajakumar A, Conrad KP. Expression, ontogeny, and regulation of hypoxia-inducible transcription factors in the human placenta. Biol Reprod **2000**; 63:559–69.
29. Mwapasa V, Rogerson SJ, Molyneux ME, et al. The effect of Plasmodium falciparum malaria on peripheral and placental HIV-1 RNA concentrations in pregnant Malawian women. AIDS **2004**; 18:1051–9.
30. Abrams ET, Brown H, Chensue SW, et al. Host response to malaria during pregnancy: placental monocyte recruitment is associated with elevated beta chemokine expression. J Immunol **2003**; 170:2759–64.
31. Abrams ET, Milner DA Jr, Kwiek J, et al. Risk factors and mechanisms of preterm delivery in Malawi. Am J Reprod Immunol **2004**; 52:174–83.
32. Boeuf P, Vigan-Womas I, Jublot D, et al. CyProQuant-PCR: a real time RT-PCR technique for profiling human cytokines, based on external RNA standards, readily automatable for clinical use. BMC Immunol **2005**; 6:5.
33. Meller M, Vadachkoria S, Luthy DA, Williams MA. Evaluation of housekeeping genes in placental comparative expression studies. Placenta **2005**; 26:601–7.
34. Vandesompele J, De Preter K, Pattyn F, et al. Accurate normalization of real-time quantitative RT-PCR data by geometric averaging of multiple internal control genes. Genome Biol **2002**; 3:RESEARCH0034.
35. Volm M, Koomagi R, Mattern J. Prognostic value of vascular endothelial growth factor and its receptor Flt-1 in squamous cell lung cancer. Int J Cancer **1997**; 74:64–8.
36. Dirkx AEM, oude Egbrink MGA, Wagstaff J, Griffioen AW. Monocyte/macrophage infiltration in tumors: modulators of angiogenesis. J Leukoc Biol **2006**; 80:1183.
37. Lewis C, Murdoch C. Macrophage responses to hypoxia: implications for tumor progression and anti-cancer therapies. Am J Pathol **2005**; 167: 627.

38. Clark DE, Smith SK, Sharkey AM, Charnock-Jones DS. Localization of VEGF and expression of its receptors Flt and KDR in human placenta throughout pregnancy. Hum Reprod **1996**; 11:1090–8.
39. Vuckovic M, Ponting J, Terman BI, Niketic V, Seif MW, Kumar S. Expression of the vascular endothelial growth factor receptor, KDR, in human placenta. J Anat **1996**; 188:361–6.
40. Kramer MS, Platt RW, Shi Wu Wen MB, et al. A new and improved population-based Canadian reference for birth weight for gestational age. Pediatrics **2001**; 108:e35.
41. Muehlenbachs A, Mutabingwa TK, Edmonds S, Fried M, Duffy PE. Hypertension and maternal/fetal conflict during placental malaria. PLoS Med **2006**; 3:e446.
42. Haddad JJ, Land SC. A non-hypoxic, ROS-sensitive pathway mediates TNF-alpha-dependent regulation of HIF-1alpha. FEBS Lett **2001**; 505: 269–74.
43. Regnault TR, de Vrijer B, Galan HL, Wilkening RB, Battaglia FC, Meschia G. Development and mechanisms of fetal hypoxia in severe fetal growth restriction. Placenta **2007**; 28:714–23.
44. Lyall F, Young A, Boswell F, Kingdom JC, Greer IA. Placental expression of vascular endothelial growth factor in placentae from pregnancies complicated by pre-eclampsia and intrauterine growth restriction does not support placental hypoxia at delivery. Placenta **1997**; 18:269–76.
45. Gurel D, Ozer E, Altunyurt S, Guclu S, Demir N. Expression of IGR-IR and VEGF and trophoblastic proliferative activity in placentas from pregnancies complicated by IUGR. Pathol Res Pract **2003**; 199:803.
46. Tse JY, Lao TT, Chan CC, Chiu PM, Cheung AN. Expression of vascular endothelial growth factor in third-trimester placentas is not increased in growth-restricted fetuses. J Soc Gynecol Investig **2001**; 8:77–82.
47. Kingdom JCP, Kaufmann P. Oxygen and placental villous development: origins of fetal hypoxia. Placenta **1997**; 18:613.
48. Arbeille P, Caries G, Tobal N, et al. Fetal flow redistribution to the brain in response to malaria infection: does protection of the fetus against malaria develop over time? J Ultrasound Med **2002**; 21:739–46.

Table 2. Immunohistochemistry scoring.

	Participants with malaria						Participants' birth weight			
	Asymptomatic (n = 10)		Without intervillositis (n = 10)		With intervillositis (n = 10)		Normal (n = 28)		Low[a] (n = 2)	
	Intensity	Consistency	Intensity	Consistency	Intensity	Consistency	Intensity	Consistency	Intensity	Consistency
Hhypoxia-inducible factor–1α										
Monocytes/neutrophils	+	++	++	++	++	+++	+	+	++	++
Syncytium	++	+++	++	+++	++	+++	++	+++	++	+++
Villus										
Stroma	–	–	–	–	–	–	–	–	–	–
Vessel										
Endothelium	++	+++	++	++	++	+++	++	++	++	+++
Lumen	–	–	–	–	–	–	–	–	–	–
Extravillous trophoblast	+++	+++	+++	+++	+++	+++	+++	+++	+++	+++
Placental growth factor										
Monocytes/neutrophils	++	++	++	+++	++	+++	+/–	+/–	+	+
Syncytium	++	+++	++	++	++	+++	+	+	++	++
Villus										
Stroma	–	–	–	–	–	–	–	–	–	–
Vessel										
Endothelium	++	+++	++	+++	++	+++	++	++	++	+++
Lumen	+	+	+	+	+	+	+/–	+/–	+	+
Extravillous trophoblast	++	+++	+++	++	+++	++	++	++	+++	+++
Vascular endothelial growth factor										
Monocytes/neutrophils	++	++	++	++	++	+++	++	+++	++	+++
Syncytium	++	+++	++	++	++	+++	++	+++	++	+++
Villus										
Stroma	+/–	+/–	+/–	+/–	+	+	–	–	+	+
Vessel										
Endothelium	++	++	+	+	++	++	+	+	+	++
Lumen	+/–	+/–	+/–	+/–	+/–	+/–	–	–	+/–	+/–
Extravillous trophoblast	+++	+++	+++	+++	+++	+++	+++	+++	+++	+++
Vascular endothelial growth factor receptor–1										
Monocytes/neutrophils	+	+	++	++	++	++	+	+	+	++
Syncytium	+	++	+	+	++	++	+	+	+	++
Villus										
Stroma	–	–	–	–	–	–	–	–	–	–
Vessel										
Endothelium	+	+	+	+	+	+	+	+	+	+
Lumen	+/–	+/–	+/–	+/–	+/–	+/–	–	–	+/–	+/–
Extravillous trophoblast	++	+++	++	++	++	++	++	+++	++	++
Vascular endothelial growth factor receptor–2										
Monocytes/neutrophils	–	–	–	–	–	–	–	–	–	–
Syncytium	–	–	–	–	–	–	–	–	–	–
Villus										
Stroma	+	+	+	+	+	+	+	+/–	+	+
Vessel										
Endothelium	++	++	++	++	++	++	+	++	++	++
Lumen	++	++	++	++	++	++	+	++	++	++
Extravillous trophoblast	–	–	–	–	–	–	–	–	–	–

NOTE. Scores are represented as follows: –, score 0; +/–, score <0.1; +, score 0.1≤1); ++, score 1≤2); +++, score >2. The only statistically significant difference between groups was a more intense (P = .037) and more consistent (P = .041) staining of vascular endothelial growth factor in the villous stroma of patients with intervillositis, compared with that in participants without intervillositis, regardless of malaria status (Mann-Whitney test).

[a] <2500 g.