Human Immunodeficiency Virus Co-Infection Increases Placental Parasite Density and Transplacental Malaria Transmission in Western Kenya

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Abstract. Plasmodium falciparum malaria and human immunodeficiency virus (HIV)-1 adversely interact in the context of pregnancy, however little is known regarding the influence of co-infection on the risk of congenital malaria. We aimed to determine the prevalence of placental and congenital malaria and impact of HIV co-infection on transplacental malaria transmission in 157 parturient women and their infants by microscopy and by quantitative real-time polymerase chain reaction (PCR) in western Kenya. The prevalence of placental and cord blood infections were 17.2% and 0% by microscopy, and 33.1% and 10.8% by PCR. HIV co-infection was associated with a significant increase in placental parasite density (P < 0.05). Cord blood malaria prevalence was increased in co-infected women (odds ratio [OR] = 5.42; 95% confidence interval [CI] = 1.90–15.47) and correlated with placental parasite density (OR = 2.57; 95% CI = 1.80–3.67). A 1-log increase in placental monocyte count was associated with increased risk of congenital infection (P = 0.001) (OR = 48.15; 95% CI = 4.59–505.50). The HIV co-infected women have a significantly increased burden of placental malaria that increases the risk of congenital infection.

INTRODUCTION

Plasmodium falciparum malaria infections are more frequent and have worse clinical outcomes in pregnant versus non-pregnant individuals. Pregnancy carries a 2- to 3-fold increased risk of malaria infection, and one in four women living in areas of stable transmission may be infected at time of delivery.1,2 Pregnancy-associated malaria (PAM) has profound maternal and fetal health consequences, including an increased risk of maternal anemia, intrauterine growth restriction (IUGR), and delivery of pre-term and low birth weight infants (LBW).3–5 Plasmodium falciparum PAM variants are able to evade innate phagocytic clearance and prior gained immunity,6,7 and accumulate in the placenta, contributing to an inflammatory response that can damage the syncytiotrophoblast membrane, a potential mechanism for transplacental transmission of parasites to the fetus.8,9

The prevalence, risk factors, and biologic implications of transplacental infection of the fetus (congenital malaria) are not well understood. Although parasites are occasionally detected in cord blood, symptomatic congenital malaria is uncommonly reported. However, exposure to malaria in utero may induce tolerance or alternatively sensitize the infant and thereby influence susceptibility to future malaria infections.10–18

Mounting evidence suggests that human immunodeficiency virus (HIV) and malaria negatively interact, with malaria driving HIV progression and transmission and vice versa.19–22 Malaria increases HIV replication in vitro19 and in vivo,23,24 conversely, HIV appears to increase the risk of clinical and severe malaria.20,24 Recent mathematical modeling data applied to western Kenya suggest that co-infection may have facilitated the spread of both diseases in sub-Saharan Africa.25

This deleterious interaction appears to be most profound in the context of pregnancy. The burden of HIV-malaria co-morbidity is particularly evident in sub-Saharan Africa where an estimated 1 million pregnancies are complicated by malaria-HIV co-infection each year.26 Malaria increases HIV viral load in pregnant women and increases CCR5 expression on intervillous maternal and fetal villous macrophages, which may increase risk for fetal infection.27,28 However, whether placental malaria increases the risk of mother to child transmission of HIV remains unclear.29–31

Conversely, HIV infection in pregnancy is associated with higher rates of clinical malaria, higher parasite density, and a higher risk of maternal anemia and LBW.32 HIV infection extends the risk of PAM from primigravid to multigravid women so that all gravidities are at risk of PAM and its associated adverse pregnancy outcomes. Whether HIV co-infection also increases the risk of congenital malaria is unknown.

Previous estimates of congenital malaria prevalence may be under-representative because of the limited sensitivity of light microscopy used in the majority of studies. Recently, the use of polymerase chain reaction (PCR) assays has been shown to overcome this limitation, and to correlate with clinical outcome.15,16,18,33,34 In particular, a standardized real-time quantitative PCR assay has been developed that provides a specific and sensitive method for quantitative analysis of parasite load in clinical specimens.33

We hypothesized that a real-time PCR assay would provide a better estimate of malaria prevalence and density than microscopy, and that this would allow for an examination of how HIV and malaria co-infection may affect the risk of congenital malaria. We carried out a cross-sectional study of 157 parturient women in western Kenya to examine the association between maternal placental malaria and cord blood parasitemia, and their association with HIV status.

MATERIALS AND METHODS

Study population. Our analysis was carried out in two study areas of western Kenya, Kisumu, and Siaya, which vary in transmission intensity. Both areas are holoendemic for malaria, however in rural areas northwest of Kisumu, transmission is
more intense even with the recent increased use of insecticide-treated bednets. A subset of women recruited into a larger study designed to assess immunoprotection and immunopathogenesis in placental malaria were randomly selected for analysis in the present study. This study was approved by the Institutional Review Boards of the University of Georgia, the Centers for Disease Control and Prevention, and the Kenya Medical Research Institute Ethical Review Committee.

In Kisumu, women were recruited when they presented for delivery at the New Nyanza Provincial General Hospital, a government referral hospital that primarily captures women from urban and peri-urban areas of Kisumu. Similarly in Siaya, parturient women were recruited at Siaya District Hospital, a smaller government hospital that serves residents of surrounding rural communities. Inclusion criteria for the parent study were singleton, uncomplicated vaginal delivery, aged 15 to 40 years with no apparent infection or underlying medical condition as determined by history and physical examination. Exclusion criteria included complicated or multiple birth, Caesarean section, age < 15 years or > 40 years of age, delivery outside of the hospital, any apparent infection (besides malaria and HIV), or underlying medical condition. Women with indeterminate HIV test results were also excluded. Following written informed consent, women were counseled and tested for HIV infection using commercially available assays, Unigold (Trinity Biotech, Bray, Ireland) and Determine HIV 1/2 (Inverness Medical Innovations, Inc., Waltham, MA) and discordant results were resolved with Capillus Rapid Test (HIV-1/HIV-2 from Trinity Biotech). They were screened for malaria by examining at least 100 oil-immersion fields of Giemsa-stained thick and thin blood smears of peripheral, placental, and cord blood as described.

**Specimen collection.** Maternal placental blood was isolated either by the prick method immediately after placental expulsion or by perfusion, with no bias between recruitment sites. After careful cleaning, cord blood was obtained by direct sampling of the umbilical vein. Maternal placental blood, and cord blood was collected into either EDTA or heparin. Peripheral blood was collected into either heparin or EDTA by venipuncture within 12 hours of delivery. Red blood cell pellets were separated and stored at −80°C until use.

**DNA extraction, real-time PCR, and flow cytometric analysis of samples.** The DNA was extracted from 200 µL of red blood cell pellet using the Qiagen DNeasy Blood and Tissue Kit (Qiagen, CA) according to the manufacturer’s protocol. A standardized commercially available quantitative real-time PCR assay (RealArt Malaria PCR assay, Artus GmbH, Germany) was used on a Lightcycler 1.5 (Roche Diagnostics, Quebec, Canada) platform and version 4.0 software, according to the manufacturer’s protocol.

Monocyte levels, as a percent of total leukocytes in freshly perfused placental blood, were estimated by flow cytometry using antibodies specific for human CD14 (clone M5E2, Pharmingen, Franklin Lakes, NJ) and CD45 (clone H130, Pharmingen) with analysis on a FACSCalibur cytometer (Becton-Dickinson, Franklin Lakes, NJ) using CellQuest software (Becton-Dickinson). Monocyte counts were calculated by multiplying percent monocytes by total white blood cell count obtained from analysis of prick blood on a Coulter Hematology Analyzer (COULTER Ac·T diff2, Bakersfield, CA).

**Statistical analysis.** The SPSS version 15.0 (Chicago, IL) was used for statistical analysis. Other than where stated, data from real-time PCR was used for analysis. Prevalence of parasitemia in peripheral and placental blood samples detected by microscopy and PCR was calculated and differences tested using Pearson’s chi-square analysis. Parasite densities and monocyte counts were log-transformed for statistical analysis and Student t test was used to compare differences between groups.

The association of exposure variables (maternal HIV infection, parity, region of delivery, and placental parasitemia) with the presence of parasitemia in cord blood was evaluated by logistic regression analysis. Univariate analysis was first carried out to test individual associations. Multivariate analysis was then carried out using covariables that were significant in univariate analysis. The multivariate model was built by the forward conditional method, with added covariables needing to significantly improve the −2 log likelihood score for inclusion. Non-linear regression of cord blood prevalence versus log-transformed stratified placental parasitemia was visualized in DataFit 8.2.X (Oakdale Engineering, Oakdale, PA).

**RESULTS**

**Participants.** Study population–specific parameters are described in Table 1. The percentage of low parity (pregnancy 1 or 2) women in Kisumu was found to be significantly higher (P < 0.05) than in Siaya, with 75% of women sampled in Kisumu and 58.5% of women sampled in Siaya being of low parity. The higher representation of low parity women in Kisumu is reflective of the status of the Provincial Hospital as a referral center, which by definition attracts younger, low order pregnant women who have a greater tendency for extended and/or difficult labor.

**Improved malaria detection by real-time PCR.** Compared with microscopy, the real-time PCR assay consistently detected a significantly higher prevalence of infection (P < 0.001, Figure 1A). On the basis of real-time PCR as the reference standard, microscopy had a sensitivity and specificity for detection of *P. falciparum* malaria in peripheral blood of 44.6% and 99.0%, and in placental blood of 48.2% and 100%, respectively. Of 157 women enrolled in the study, 56 (35.7%) and 52 (33.1%) women were positive for placental and peripheral malaria, respectively, as determined by real-time PCR compared with 27 (17.2%) and 25 (15.9%) by microscopy (P < 0.01). Cord blood analysis found 17 samples (10.8%) positive for malaria by PCR, whereas none (0%) were detected positive by smear (P < 0.01). All but one of the cord blood samples that were positive by real-time PCR had less than 100 genome copies/µL. Microscopy and real-time PCR

| Table 1 |  |
|---|---|---|
| Kisumu and Siaya population specific parameters | Total | Kisumu | Siaya |
| Maternal characteristics | | |
| N = 157 | N = 92 | N = 65 |
| Age (years) | | |
| < 20 | 30.1% | 32.5% | 26.1% |
| 20–29 | 58.5% | 57.1% | 60.9% |
| > 29 | 11.4% | 10.4% | 13.0% |
| Gravidity | | |
| G1 | 43.9% | 52.2% | 32.3% |
| G2 | 24.2% | 22.8% | 26.2% |
| G3 | 15.9% | 10.9% | 23.1% |
| G4+ | 15.9% | 14.1% | 18.5% |
| HIV status | | |
| HIV+ | 24.8% | 22.8% | 27.7% |
| HIV− | 74.5% | 76.1% | 72.3% |
The prevalence of peripheral and cord blood infection were also significantly higher in Siaya compared with Kisumu (47.7% versus 22.8% and 20% versus 4.4%, respectively; \( P < 0.01 \)). Overall compared with Kisumu, women in Siaya had an odds ratio (OR) of 3.52 (95% confidence interval [CI] = 1.79–6.91) of having peripheral or placental malaria and 5.50 (95% CI = 1.70–17.76) of having cord blood parasitemia (Figure 2).

**Placental malaria risk decreases with successive pregnancies.** Logistic regression analysis was carried out to examine the risk of placental malaria versus parity (pregnancy count) while controlling for region of origin. Pregnancy count \( (P < 0.05) \) and region of origin \( (P < 0.001) \) were found to be significant exposure variables in determining risk of placental malaria in a multivariate model. Increasing pregnancy count had an OR of 0.76 (95% CI = 0.58–0.99), whereas the region-dependent OR was 4.19 (95% CI = 2.04–8.62), with women residing in Siaya carrying the increased risk (Figure 2). The prevalence of placental malaria for each region was plotted against parity to visualize the significant trends (Figure 3).

**Impact of HIV co-infection on placental parasite burden.** To explore the hypothesis that HIV infection might increase the risk of placental and cord blood malaria, we first examined the effect of co-infection on placental parasitemia. We observed that HIV co-infected women had a significantly increased burden of placental malaria (Figure 4). Placental parasite density was found to be significantly higher in HIV-positive individuals \( (8.46 \times 10^3 \text{ genome copies/µL} \text{ versus } 0.57 \times 10^3 \text{ genome copies/µL}, \ P < 0.05) \). The prevalence of placental malaria also increased in HIV co-infected women from 33.3% to 43.6%, but this did not reach statistical significance \( (P = 0.25) \).

Regionally, co-infected women in Siaya experienced a more marked increase in placental parasite density. Although HIV-negative women in Kisumu and Siaya had similar mean placental parasite burdens of 573 and 340 genome copies/µL \( (P = 0.99) \), co-infected women residing in Siaya had a notable 33-fold increase in placental parasite density with a mean of 11,200 genome copies/µL \( (P < 0.001) \). Co-infected women in Kisumu also displayed an increase in placental parasite burden (7.5-fold), but this did not reach statistical significance \( (P = 0.35) \).

**Impact of HIV and placental parasite burden on risk of cord blood malaria.** Because HIV status was found to substantially increase placental parasite density, we next examined the association of these factors with a presence of malaria in cord blood. By univariate analysis both HIV status \( (P = 0.02) \) and placental parasitemia \( (P < 0.001) \) were significant predictors of cord parasitemia, with an OR 5.42 (95% CI = 1.90–15.47), and 2.57 (95% CI = 1.80–3.67), respectively (Figure 2).
the effect of HIV co-infection was stronger in Siaya, where it was associated with an OR of 10.75 (95% CI = 2.71–42.72) for cord parasitemia.

Multivariate regression analysis was next applied to test whether HIV status had an effect on cord malaria independent of its interaction with placental parasitemia. Multivariate analysis showed an improvement in predictive power over either univariate analysis, however HIV infection became borderline non-significant in multivariate analysis (adjusted OR 4.36; 95% CI 0.99–16.73; P = 0.051).

As placental parasite burden was found to be a highly significant determinant of cord blood malaria, this relationship was modeled by plotting prevalence of cord infection against stratified log-transformed placental parasitemia, using only the placental positive women from Siaya (Figure 4C). This distinguished 6 groups, from 1 to 10 genome copies/µL in group 1, 1 × 10^1 – 9.99 × 10^1 genome copies/µL in group 2, and so on. The function obtained was then applied to HIV-stratified cases of placental parasitemia in Siaya and Kisumu. Although HIV-infected women in Siaya were predicted to be at a high risk of cord parasitemia, the model also predicted that the HIV-dependent increase in placental parasitemia of Kisumu women was not substantial enough to increase their risk of cord malaria, as was observed (Figure 4D).

**Risk of cord blood malaria is associated with placental parasite burden and monocyte count.** Placental monocytes contribute to altered cytokine profiles and elevated pro-inflammatory cytokine levels that may be important mediators of placental tissue injury and poor birth outcomes associated with PAM. Therefore, we hypothesized that placental parasitemia might determine risk of cord parasitemia in part by recruitment of monocytes to the placenta. Flow cytometry was used to determine monocyte counts in a subset of randomly chosen placental blood samples (N = 62), allowing for an analysis of correlation between mononuclear cells, placental parasitemia and cord malaria. Regression analysis of log monocyte count versus log placental parasite burden revealed a highly significant, direct correlation (P < 0.001, Figure 5A). Supporting this observation, women with placental parasitemia had a higher mean monocyte count than women without placental parasitemia (2647.9 versus 1527.0, P = 0.02, Figure 5B). Univariate analysis was then performed on log-transformed monocyte counts with cord malaria as the outcome. Notably, a 1-log increase in placental monocyte count significantly increased risk of cord parasitemia (P = 0.001) with an odds ratio of OR 48.15 (95% CI = 4.59–505.50, Figure 2). However, on multivariate analysis, after adjustment for placental parasitemia, monocyte count was no longer an independent predictor of cord parasitemia.

**DISCUSSION**

In this study, Giemsa-stained blood films and a standardized real-time PCR assay were used to detect malaria in peripheral, placental, and cord-blood from pregnant women and their infants in western Kenya. Here we report a high
prevalence of malaria present in cord blood (10.8%) and show that HIV co-infection increased placental parasite density and the rate of antenatal malaria transmission. Furthermore, we also found a highly significant correlation between the number of monocytes in placental blood and placental malaria, and cord parasitemia. The significance of PCR-detectable parasite DNA in microscopically negative cord bloods is uncertain. Positive detection of malaria in cord blood could result from contamination with maternal blood during sampling, or could be representative of transplacental malaria transmission. Malhotra and others have demonstrated discordant genotyping between maternal peripheral malaria and those detected in cord blood, which strongly supports the notion that congenital malaria does occur. Although it is not possible to determine what proportion of cord malaria cases are caused by contamination at birth, the prevalence reported here (10.8%) is comparable to that reported by Malhotra using similar methods (10.4%).

We compared malaria detection by a real-time PCR assay, which accurately and quantitatively determines malaria burden versus microscopic blood smear analysis (Figure 1A). The PCR analysis consistently detected significantly higher rates of peripheral, placental, and cord blood infection than microscopy. This may be due in part to enhanced detection of low-level parasitemia by real-time PCR assays. For instance, the average parasitemia measured in all cord-blood positive samples in this study was 21 genome copies/µL; none of these cases were detected by conventional microscopy. The limited sensitivity of microscopy combined with the high prevalence rate when more sensitive detection methods are used, suggests that congenital malaria may have a broad but under-appreciated public health impact.

We observed a high prevalence of placental malaria infection within our study population (Figure 1B). Women in Siaya had a placental infection prevalence rate of 52.3%, notably higher than women in Kisumu (23.9%; P < 0.001) whose infection rates were more in line with other endemic areas. Multivariate logistic regression analysis was applied to test the dependence of placental malaria infection on pregnancy count, and found that successive pregnancies produced a decreased risk with an odds ratio of 0.76 (P = 0.04, Figures 2 and 3).

The prevalence of congenital malaria, defined by the positive detection of malaria in cord blood by real-time PCR, was 10.8% in the total sampled population, with Kisumu and Siaya having a rate of 4.4% and 20% of antenatal transmission, respectively. The observed prevalence rates are consistent with a comparable study that used PCR to determine the prevalence of cord blood malaria in coastal Kenya that also used PCR to determine the prevalence of cord blood malaria.

We next examined the relationship between cord parasitemia and placental parasite burden by logistic regression analysis. It was found that placental parasite density was highly predictive of cord blood positive cases. A 1-log increase in placental parasite burden carried an increased risk for congenital malaria of OR 2.57 (95% CI = 1.80–3.67; P < 0.001). The HIV serostatus was also found to strongly correlate with cord blood infection, however the significance became borderline when controlling for placental parasitemia, suggesting that HIV may impact congenital malaria primarily by allowing for higher parasite densities in the placenta.

Among those women with placental malaria infections, HIV co-infected women had a 14.8-fold increase (P < 0.05) in parasite burden over HIV-negative women. This increase was particularly marked in Siaya with a 33-fold difference (P < 0.001). On the basis of data from Siaya, we generated a model for predicting risk of cord malaria based on placental parasitemia (Figure 4C) and applied it to women from Kisumu. Stratified by HIV serostatus, it predicted that the marginal increase in placental parasitemia of co-infected women in Kisumu should not significantly impact their risk of congenital malaria (Figure 4D). Comparison of predicted and measured rates of congenital malaria supported the relationship between HIV status, placental parasitemia, and risk of congenital malaria.

It has been demonstrated previously that recruitment of monocytes to the placenta occurs during PAM. It has also been suggested that damage to the placenta resulting from a monocyte-associated inflammatory response could...
increase the risk of antenatal malaria transmission.\textsuperscript{13,14} We examined monocyte counts by flow cytometry in a subset of randomly selected placental blood samples and found a significant and direct correlation between monocyte count and placental parasitemia (Figure 5). We then tested whether monocyte count was a significant predictor of cord blood malaria. Notably, a 1-log increase in monocyte count was found to increase the OR of cord malaria by 48.15 (95\% CI = 4.59–505.50; Figure 2). It is therefore possible that the host’s immunopathological interaction with malaria in the placenta provides a mechanism by which transplacental malaria transmission can occur.

Previous studies support a significant interaction between HIV and malaria particularly during pregnancy. However, the risk factors for perinatal transmission of malaria are poorly understood and are likely to be complex. There are several potential explanations for why malaria and HIV co-infection may have resulted in a higher prevalence of congenital malaria in Siaya than Kisumu. Exposure to malaria may occur more frequently in Siaya resulting in more frequent infections and higher parasite densities in HIV-infected women. Although rates of HIV were similar between the two tested sites, there may be a discrepancy between the two locations regarding the degree of HIV disease progression. In a previous study, Ayisi and others found that high placental parasitemia, high HIV viral load, and low CD4+ counts are associated with vertical transmission of HIV, supporting an important interaction between these diseases.\textsuperscript{20} It would therefore be relevant to examine the correlation between placental parasite burden, CD4+ cells and HIV viral load in determining cord blood parasitemia, as viral load and immune status could be strong determinants of congenital malaria.

In summary, we describe a dependence of antenatal transmission of malaria on placental parasite density and on recruitment of inflammatory cells to the placenta. Placental and cord parasitemia were found to be significantly increased in HIV-infected women. The rate of congenital malaria was notably high in Siaya, Kenya, where a high prevalence, placental parasite burden, and HIV combine to represent a major public health burden during pregnancy.

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