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Maternal–Fetal Microtransfusions and HIV-1 Mother-to-Child Transmission in Malawi

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Author Contributions: JJK participated in hypothesis generation, data collection, and data analysis, and was the primary author of this study. VM participated in the study design, data collection, and the writing of the manuscript. DAM diagnosed chorioamnionitis. APA contributed to the data analysis. WCM suggested the case-cohort design and participated in data analysis and manuscript preparation. ET contributed to data collection and was the Chief Obstetrician. MEM participated in participant recruitment and study management/supervision. SJR diagnosed placental malaria, developed the hypothesis, participated in data analysis, and provided study oversight. SRM developed the hypothesis; participated in the study design, data analysis, and study coordination; and was the Principal Investigator. All of the authors edited and revised the final manuscript.

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Abbreviations: CI, confidence interval; IP, intrapartum; IU, in utero; MHP, Malaria and HIV in Pregnancy; MTCT, mother-to-child transmission; PLAP, placental alkaline phosphatase; RR, risk ratio

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ABSTRACT

Background

Between 25% and 35% of infants born to HIV-infected mothers become HIV-1 infected. One potential route of mother-to-child transmission (MTCT) could be through a breakdown in the placental barrier (i.e., maternal–fetal microtransfusions).

Methods and Findings

Placental alkaline phosphatase (PLAP) is a 130-kD maternal enzyme that cannot cross the intact placental barrier. We measured PLAP activity in umbilical vein serum as an indicator of maternal–fetal microtransfusion, and related this to the risk of HIV-1 MTCT. A case-cohort study was conducted of 149 women randomly selected from a cohort of HIV-1-infected pregnant Malawians; these women served as a reference group for 36 cases of in utero MTCT and 43 cases of intrapartum (IP) MTCT. Cord PLAP activity was measured with an immunocatalytic assay. Infant HIV status was determined by real-time PCR. The association between cord PLAP activity and HIV-1 MTCT was measured with logistic regression using generalized estimating equations. Among vaginal deliveries, PLAP was associated with IP MTCT (risk ratio, 2.25 per log_{10} ng/ml PLAP; 95% confidence interval, 0.95–5.32) but not in utero MTCT. In a multivariable model adjusted for HIV-1 RNA load, chorioamnionitis, and self-reported fever, the risk of IP MTCT almost tripled for every log_{10} increase in cord PLAP activity (risk ratio, 2.87; 95% confidence interval, 1.05–7.83).

Conclusion

These results suggest that during vaginal deliveries, placental microtransfusions are a risk factor for IP HIV-1 MTCT. Future studies are needed to identify factors that increase the risk for microtransfusions in order to prevent IP HIV-1 MTCT.
Introduction

By the end of 2004, sub-Saharan Africa was home to 13.3 million HIV-1-infected women, and in many sub-Saharan countries, the HIV-1 prevalence in antenatal women exceeded 20% [1]. In the absence of interventions such as antiretroviral drug prophylaxis, elective cesarean sections, and replacement feeding, between 25% and 35% of the children born to HIV-1-positive women themselves become infected [2]. Of the infant infections, approximately one-half occur during labor and delivery through an unknown mechanism [3].

One potential mechanism of HIV-1 mother-to-child transmission (MTCT) is through direct contact of infant mucosa with HIV-1-infected maternal blood, amniotic fluid, or cervical/vaginal secretions (the “all mucosal” mechanism) [4]. Evidence for the importance of this mechanism during intrapartum (IP) MTCT includes the observations that elective cesarean sections reduce HIV-1 MTCT [5], and that higher quantities of HIV-1 secreted into the birth canal are associated with increased HIV-1 MTCT [6,7]. On the other hand, both birth canal disinfection and emergency cesarean sections reduce birth canal exposure to HIV-1, but neither intervention significantly reduces HIV-1 MTCT [5,8-11].

In addition to the “all mucosal” hypothesis, HIV-1 MTCT could also occur via a breakdown in the maternal-fetal barrier followed by placental microtransfusions. Placental microtransfusions have previously been suggested as a route of HIV-1 transmission [12-15], and they are considered a plausible route of hepatitis B, C, and G vertical transmission [16-18]. Recently, an assay has been developed to quantify placental microtransfusions based on the detection of placental alkaline phosphatase (PLAP) activity in umbilical cord serum [13,15]. PLAP is a 130-kD glycoprotein synthesized from the eighth week of gestation until parturition, and its large molecular size is believed to preclude its passive diffusion across the placental barrier. Based on these characteristics and the observation that infants produce small amounts of PLAP compared to pregnant women, cord PLAP can be used as a surrogate marker of placental microtransfusions [13].

Although the cause of placental microtransfusions is unknown, they are hypothesized to arise during the first stage of labor, when uterine contractions intensify and membranes rupture [3,12,13]. In support of this hypothesis, cesarean sections performed before the onset of labor result in significantly less cord PLAP activity than both emergency cesarean deliveries and spontaneous vertex deliveries [13,15]. To build on these observations and to expand our knowledge of the biological mechanism of HIV-1 MTCT, we measured cord PLAP activity in Malawian mother-offspring pairs and evaluated placental microtransfusions as a risk factor for HIV-1 MTCT.

Methods

Participant Recruitment

This case-cohort study was derived from the Malaria and HIV in Pregnancy (MHP) prospective cohort study that was approved by the College of Medicine Research Committee at the University of Malawi and the Institutional Review Boards of the University of Michigan and the University of North Carolina at Chapel Hill. From December 2000 until March 2004, women in the Antenatal Ward at Queen Elizabeth Central Hospital in Blantyre, Malawi, were screened for eligibility to participate in a prospective cohort study designed to determine the association between malaria and HIV-1 MTCT. Women were ineligible for the study if they were in the active phase of labor, were participating in other research studies, lived outside the Blantyre district, were less than 15 y of age, were hypertensive, or had altered consciousness. Consententing women received HIV pre- and post-test counseling, and all HIV-1-infected women and their offspring received nevirapine according to the HIVNET 012 protocol [19]. The association between malaria and HIV-1 viral load in the MHP cohort has been described previously [20].

Case-Cohort Design

We constructed a case cohort study from the parent MHP prospective cohort. With this study design, a rare disease assumption is unnecessary and the odds ratio provides a direct estimate of the risk ratio (RR) [21]. Between December 2000 and February 2003, 2,575 pregnant women were recruited in the MHP study, of whom 744 (29.1%) were HIV infected. After identifying all cases of in utero (IU) and IP MTCT in the parent cohort, we randomly selected 160 women, regardless of their HIV-1 transmission status, from the 744 HIV-1-positive women to serve as a reference cohort. Of these mother-offspring pairs, three were excluded because of multiple gestations, six were excluded because they delivered at home (therefore cord serum was unavailable), and two were excluded because the mother died during delivery; the remaining 149 HIV-infected women formed the reference cohort of the case-cohort study (Figure 1). All cases of IU MTCT were selected from women enrolled in the MHP study through February 2003, and all cases of IP MTCT were selected from women enrolled in the MHP study through June 2003. IP transmission cases selected from February to June 2003 did not differ from the other IP transmission cases in terms of age, peripheral HIV-1 RNA load, PLAP activity, hemoglobin concentration, placental malaria infection, or mode of delivery (data not shown). HIV-1 MTCT cases were considered IU transmissions if the infant was positive for HIV-1 DNA within 48 h of birth, and were considered IP transmissions (i.e., HIV-1 infection occurring at or around the time of delivery) if the infant was both negative for HIV-1 DNA
DNA within 48 h of birth and positive for HIV-1 DNA 6 wk after delivery [22].

Laboratory/Pathology Testing

Chorioamnionitis was assessed by a board-certified pathologist (DM) according to the methods described in [23]. Peripheral malaria infection was assessed on thick blood films stained with field stain. Placental malaria was diagnosed from formalin-fixed placental biopsies as described in [24]. Maternal hemoglobin concentration was determined by HemoCue hemoglobinometer (HemoCue, Ängelholm, Sweden), and CD8-positive T cells were quantified by FACScan (Becton Dickinson, San Jose, California, United States).

HIV-1 Testing

Within the MHP cohort, maternal HIV-1 status was determined concurrently with both the Determine HIV-1 Rapid Test (Abbott Laboratories, Abbott Park, Illinois, United States) and the SeroCard HIV-1/2 Rapid Test (Trinity Biotech, Bray, Ireland). HIV-1 RNA was quantified using Amplicor HIV-1 Monitor v1.5 (Roche Diagnostics, Branchburg, New Jersey, United States), with plasma HIV-1 RNA concentrations less than 400 copies/ml assigned a value of 400 copies/ml. Infants were considered HIV-1 infected based on the detection of HIV-1 DNA with a real-time PCR assay against the HIV-1 long terminal repeat [25]. Maternal HIV-1 proviral load was quantified according to the methods presented in [6].

PLAP Assay

Immediately after delivery the umbilical cord was clamped and cut. A section of the umbilical cord 3 cm from its point of insertion into the placenta was washed with saline, the umbilical vein was located, and cord blood was aspirated with a large-bore needle. Serum was prepared from the blood and stored at −80 °C. PLAP was isolated from 100 μl of cord serum using an isoform-specific anti-PLAP antibody (clone B431, Biomeda, Foster City, California, United States), and its fluorescence was measured on a PerkinElmer (Wellesley, Massachusetts, United States) fluorimeter (excitation λ = 490 nm, 10-nm slit width; emission λ = 514 nm, 2.5-nm slit width, 515-nm cutoff filter). Cord PLAP concentration was determined by interpolation from a standard curve of purified human PLAP (Sigma, St. Louis, Missouri, United States). The standard curve was linear from 12 ng/ml to 3,250 ng/ml ($R^2 = 0.992$), with PLAP activity less than 12 ng/ml assigned a value of 12 ng/ml.

### Statistical Methods

IU and IP MTCT cases were analyzed as independent outcomes. We used generalized estimating equations with a logit link, binomial distribution, and an independent correlation structure to conduct bivariable and multivariable assessments of the relationship between maternal features and MTCT, while accounting for the lack of independence of the 23 MTCT cases included in the sub-cohort. To reflect the monotonically increasing risk of IP transmission as PLAP increased, log$_{10}$ cord PLAP was coded as a continuous variable. In the multivariable models, prior to the outcome analysis, we assessed heterogeneity of the odds ratio by testing for interaction terms at $\alpha = 0.1$. Subsequently, confounding was assessed by the backward elimination method [21] based on the change in the point estimate; variables that changed the estimate (RR) more than 10% were retained in the final model, with the exception of maternal HIV-1 viral load, which was included a priori. Variables assessed for interaction and potential confounding of HIV-1 MTCT prior to the outcome analysis include the following: HIV-1 RNA load, HIV-1 DNA load, CD4 T cell count < 200 cells/μl, chorioamnionitis (present or absent), episiotomy, self-reported fever in the week prior to enrollment, gestational age, placental malaria (any *Plasmodium falciparum*-infected erythrocytes on placental histology), and rupture of membranes more than 4 h prior to delivery. Owing to the correlation between HIV-1 RNA load, HIV-1 DNA load, and CD4 T cell count, the multivariable model of MTCT contained only one of these measures of HIV burden. Before the analysis of the association between maternal features and log$_{10}$ cord PLAP, we eliminated duplicate values by removing the 23 cases included in the sub-cohort. The relationships between PLAP and dichotomous maternal factors were assessed with a two-tailed unpaired $t$-test ($\alpha = 0.05$), and with continuous maternal factors the relationship was assessed with Pearson’s correlation coefficient ($\alpha = 0.05$). Statistical analysis was performed with STATA v8.2 (StataCorp, College Station, Texas, United States).

### Results

We designed a case-cohort study to independently compare 36 IU and 43 IP MTCT cases to a sub-cohort of 149 HIV-positive mothers. The IU and IP MTCT cases were similar to the sub-cohort in terms of maternal age, infant birth weight, maternal hemoglobin concentration, gestational age, and gravidity (Table 1). The groups did not differ in the proportion of chorioamnionitis infections, nor in the proportion of peripheral or placental malaria infections. Of 205 deliveries, 149 (73%) were spontaneous vertex deliveries, 40 (20%) were emergency cesarean sections, seven (5%) were instrumental vaginal deliveries, five (2%) were elective cesarean sections, and four (2%) were breech deliveries; the mode of delivery did not differ significantly between study groups.

As expected, HIV-1 RNA concentration was associated with MTCT. The median HIV-1 RNA load of the mother in IU MTCT cases was twice the median HIV-1 RNA load of the sub-cohort (67,646 copies/ml versus 35,241 copies/ml), and in a univariable regression, the risk of IU transmission increased 65% for every log$_{10}$ increase in HIV RNA load (RR, 1.65; 95% confidence interval [CI], 1.06–2.58). Similarly, the median HIV-1 RNA load of the mother in IP MTCT cases was approximately twice the median RNA load of the sub-cohort (76,544 copies/ml versus 35,241 copies/ml), and the risk of IP MTCT increased 76% per log$_{10}$ increase in HIV-1 RNA (RR, 1.76; 95% CI, 1.10–2.81). No statistically significant association between MTCT and either HIV DNA level or CD4 count was observed.

To validate PLAP as a marker for placental microtransfusions, we determined whether cord PLAP was associated with mode of delivery and gestational age. Cord serum was available for 177/205 (87%) of the enrolled mother–offspring
Table 1. Enrollment Characteristics

<table>
<thead>
<tr>
<th>Characteristic</th>
<th>Subcategory</th>
<th>Sub-Cohort (n = 149)</th>
<th>IU MTCT (n = 36)</th>
<th>IP MTCT (n = 43)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Age, median (25th–75th percentile)</td>
<td>24 (20–28)</td>
<td>23 (21–26)</td>
<td>24 (21–29)</td>
<td></td>
</tr>
<tr>
<td>Birthweight &lt; 2.5 kg</td>
<td>37/149 (25)</td>
<td>11/36 (31)</td>
<td>12/43 (28)</td>
<td></td>
</tr>
<tr>
<td>CD4 T cells (cells/µl)</td>
<td>37/142 (26)</td>
<td>7/34 (21)</td>
<td>16/43 (37)</td>
<td></td>
</tr>
<tr>
<td>&lt;200</td>
<td>200–499</td>
<td>17/34 (50)</td>
<td>17/43 (40)</td>
<td></td>
</tr>
<tr>
<td>≥500</td>
<td>32/142 (23)</td>
<td>10/34 (29)</td>
<td>10/43 (23)</td>
<td></td>
</tr>
<tr>
<td>Chorioamnionitis</td>
<td>34/125 (27)</td>
<td>13/36 (36)</td>
<td>15/39 (38)</td>
<td></td>
</tr>
<tr>
<td>Preterm deliveries (&lt;37 wk)</td>
<td>32/144 (22)</td>
<td>11/35 (31)</td>
<td>10/42 (24)</td>
<td></td>
</tr>
<tr>
<td>Gravidity</td>
<td>1</td>
<td>41/149 (28)</td>
<td>14/36 (39)</td>
<td></td>
</tr>
<tr>
<td></td>
<td>2–4</td>
<td>91/149 (61)</td>
<td>20/36 (56)</td>
<td></td>
</tr>
<tr>
<td></td>
<td>&gt;4</td>
<td>17/149 (11)</td>
<td>2/36 (6)</td>
<td></td>
</tr>
<tr>
<td>Hemoglobin (g/dl), median (25th–75th percentile)</td>
<td>10.9 (9.7–12.0)</td>
<td>10.8 (10.1–12.7)</td>
<td>10.9 (11.3–11.5)</td>
<td></td>
</tr>
<tr>
<td>HIV DNA load (log, copies/m)</td>
<td>35,241 (10,711–126,205)</td>
<td>67,646 (13,975–177,458)</td>
<td>76,544 (25,791–218,950)</td>
<td></td>
</tr>
<tr>
<td>Mode of delivery</td>
<td>Elective cesarean section</td>
<td>4/149 (3)</td>
<td>7/36 (20)</td>
<td></td>
</tr>
<tr>
<td></td>
<td>Emergency cesarean section</td>
<td>32/149 (21)</td>
<td>6/36 (17)</td>
<td></td>
</tr>
<tr>
<td></td>
<td>Vaginal (instrumental)</td>
<td>5/149 (3)</td>
<td>2/36 (6)</td>
<td></td>
</tr>
<tr>
<td></td>
<td>Vaginal (breech)</td>
<td>3/149 (2)</td>
<td>1/36 (3)</td>
<td></td>
</tr>
<tr>
<td></td>
<td>Vaginal (spontaneous vertex)</td>
<td>105/149 (70)</td>
<td>34/36 (79)</td>
<td></td>
</tr>
<tr>
<td>Peripheral malaria</td>
<td>None</td>
<td>14/149 (9)</td>
<td>2/36 (6)</td>
<td></td>
</tr>
<tr>
<td>Placental malaria</td>
<td>Current</td>
<td>75/134 (56)</td>
<td>18/32 (56)</td>
<td></td>
</tr>
<tr>
<td></td>
<td>Past</td>
<td>24/143 (18)</td>
<td>5/32 (16)</td>
<td></td>
</tr>
<tr>
<td></td>
<td></td>
<td>35/134 (26)</td>
<td>9/32 (28)</td>
<td></td>
</tr>
</tbody>
</table>

Data are n/total (%) unless listed otherwise.

*RR = 1.11 (95% CI 0.90–1.33) per log increase in HIV DNA (copies/m).

**RR = 1.63 (95% CI 1.00–2.66) per log increase in HIV RNA (copies/ml).

***RR = 1.76 (95% CI 1.16–2.68) per log increase in HIV RNA (copies/ml).

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Discussion

In this case-cohort study of Malawian mother–offspring pairs, we tested the hypothesis that placental microtransfusions, as measured by cord PLAP activity, are an additional mechanism of HIV-1 MTCT. In support of this hypothesis, our data show that for every log10 increase in cord PLAP, the risk of IP transmission during vaginal deliveries almost tripled. Importantly, this increased risk of transmission remained after adjustment for HIV-1 RNA load, which is
the most consistently reported risk factor for HIV-1 vertical transmission (reviewed in [27]).

In contrast to IP transmission during vaginal deliveries, placental microtransfusions were not significantly associated with IU HIV-1 transmission. The lack of association between IU MTCT and placental microtransfusions may result from the short half-life of PLAP in infants (~5 d) [13]; therefore, because PLAP activity is measured at the time of delivery, PLAP that passed into fetal circulation in the weeks prior to parturition might not persist until the time of delivery, and an association with IU MTCT, if it existed, would be missed.

Although a possible inverse relationship between PLAP and IP MTCT during emergency cesarean sections was observed, the small number of IP transmission cases in this stratum (n = 6) and the wide confidence intervals of this association (95% CI, 0.04–2.68) preclude a reliable conclusion.

The cord PLAP measurements in this study are consistent with previous studies in two ways. First, gestational age was directly correlated with cord PLAP activity, which is a result of the increase in maternal PLAP production over the course of a pregnancy [28]. Second, compared to both vaginal and emergency cesarean section deliveries, elective cesarean sections produced the smallest amount of microtransfusion [13,15]. Because only elective cesarean sections eliminate labor, the relationship between mode of delivery and cord PLAP activity has been attributed to a disruption of the placental barrier by labor and contractions. In support of this theory, Kaneda and colleagues reported a direct correlation between cord PLAP activity and prolonged labor (≥5 h) [13]. We did not observe a similar correlation among women who entered labor, and this could be explained in two ways: recording the duration of labor was not the primary concern of the study, so it is possible that our measurements were imprecise, or other features of labor such as the frequency and/or intensity of contractions may more strongly influence maternal-fetal transfusions.

Besides labor, placental infection or inflammation could also compromise the maternal-fetal barrier. A common source of placental pathology in sub-Saharan Africa is malaria, and although 17% of the women in this study had active placental malaria at the time of delivery, we detected no association between cord PLAP and placental malaria. A second potential source of placental inflammation is chorioamnionitis, which has been associated with HIV-1 MTCT [29]. Although 30% of the women in this study had chorioamnionitis, it also was not associated with cord PLAP.

Based on these observations, there is no evidence from this study that placental malaria or chorioamnionitis increases placental microtransfusions.

The most probable confounding factor in this study is gestational age, which has been associated with both HIV transmission and maternal PLAP concentration. However, although the gestational age differed between the case-cohort groups, inclusion of gestational age in our IP transmission model did not change the risk estimate.

Two potential sources of uncertainty in our RR estimate are the use of PLAP as a marker of placental microtransfusions and the misclassification of HIV-1 transmission. Any measurement error in the PLAP assay (exposure) is likely to be nondifferential among the cases and the sub-cohort. Owing to the timing of infant blood collection (48 h and 6 wk after delivery), it is also possible that some of the HIV-1 transmission classified as IP was actually acquired late IU or during early breastfeeding [3]. This misclassification should be independent of PLAP activity, and therefore our RR estimate is most likely biased towards the null [21].

In this study investigating the role of maternal-fetal microtransfusions in HIV-1 MTCT, our data suggest that, independent of maternal HIV-1 viral load, placental microtransfusions during spontaneous vaginal deliveries increase the risk of IP HIV-1 MTCT. Future studies on the etiology of placental microtransfusions should provide greater insight into the mechanism of HIV-1 MTCT and suggest new strategies to prevent IP HIV-1 transmission in the developing world.

Acknowledgments

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References


Table 2. Risk of IP HIV-1 MTCT during Vaginal Delivery per log_{10} Increase in Cord PLAP

<table>
<thead>
<tr>
<th>IP MTCT</th>
<th>RR</th>
<th>95% CI</th>
<th>CLR*</th>
<th>p-Value</th>
</tr>
</thead>
<tbody>
<tr>
<td>Crude</td>
<td>2.25</td>
<td>0.95–5.32</td>
<td>5.60</td>
<td>0.066</td>
</tr>
<tr>
<td>Adjusted for HIV RNA</td>
<td>2.01</td>
<td>0.84–4.79</td>
<td>5.70</td>
<td>0.116</td>
</tr>
<tr>
<td>Multivariable adjusted (HIV RNA)*</td>
<td>2.87</td>
<td>1.05–7.83</td>
<td>7.45</td>
<td>0.039</td>
</tr>
<tr>
<td>Multivariable adjusted (HIV DNA)*</td>
<td>3.73</td>
<td>1.27–11.0</td>
<td>8.70</td>
<td>0.017</td>
</tr>
<tr>
<td>Multivariable adjusted (CD4 T Cells &lt; 200)*</td>
<td>3.35</td>
<td>1.22–9.16</td>
<td>7.45</td>
<td>0.018</td>
</tr>
</tbody>
</table>

* CLR: confidence limit ratio — upper 95% confidence limit/lower 95% confidence limit.
*Multivariable models are adjusted for self-reported fever, chorioamnionitis, and the measure of HIV-1 burden listed in the parentheses.

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