MAJOR ARTICLE

Longitudinal Analysis of Human Immunodeficiency Virus Type 1 RNA in Breast Milk and of Its Relationship to Infant Infection and Maternal Disease

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Transmission of human immunodeficiency virus type 1 (HIV-1) via breast-feeding can occur throughout lactation. Defining both fluctuation in breast-milk virus level over time and how breast-milk virus correlates with mother-to-child transmission is important for establishing effective interventions. We quantified breast-milk HIV-1 RNA levels in serial samples collected from 275 women for up to 2 years after delivery. Higher maternal plasma virus load, lower maternal CD4 T cell count, and detection of HIV-1 DNA in maternal genital secretions were significantly associated with elevated breast-milk HIV-1 RNA. Within women who breast-fed, median virus load in colostrum/early milk was significantly higher than that in mature breast milk collected 14 days after delivery ($P \leq .004$). Breast-feeding mothers who transmitted HIV-1 to their infants had both significantly higher breast-milk viral RNA throughout lactation and more-consistent viral shedding, compared with mothers who did not transmit HIV-1. In breast-feeding women, a 2-fold–increased risk of transmission was associated with every 10-fold increase in breast-milk virus load (95% confidence interval, 1.3–3.0; $P < .001$). These results indicate that the risk of infant infection from breast-feeding is influenced by breast-milk virus load, which is highest early after delivery.

During 2001, ~800,000 children worldwide acquired human immunodeficiency virus type 1 (HIV-1), 90% via mother-to-child transmission [1]. In the absence of treatment, approximately one-third to one-half of HIV-1 infections in infants occur via breast-feeding [2–5]. Although antiretroviral therapy has been effective in reducing perinatal transmission of HIV-1, no therapeutic regimen has yet been developed that, throughout lactation, significantly reduces transmission via breast-feeding [6–12]. In developing countries, where 90% of HIV-1–exposed children live, alternatives to breast-feeding are often unaffordable, and, in settings where clean water in unavailable, their use may cause an increased susceptibility to other infectious diseases. Therefore, breast-feeding, regardless of maternal HIV-1 infection status, continues to be the most prevalent form of infant feeding in resource-constrained settings.

Antiretroviral drugs reduce transmission of HIV-1, presumably by decreasing its levels in both maternal

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blood and maternal mucosal secretions, resulting in reduced infant exposure to the virus. It is possible that antiretroviral therapy given to the mother during the breast-feeding period might be effective in preventing transmission of HIV-1 via breast milk; however, it is not known how much a reduction in breast-milk virus levels would affect the risk of transmission, nor is it known whether there is an optimal time to administer antiretroviral therapy that, throughout lactation, would prevent transmission via breast feeding. To better understand how antiretroviral therapy might prevent transmission via breast milk, the association between breast-milk virus levels and transmission of HIV-1, as well as the fluctuation in breast-milk virus load throughout the period of lactation, need to be well characterized.

Previous studies have indicated that HIV-1 is present in breast milk and that breast-milk virus levels are associated with mother-to-child transmission [13–16]. A study of 334 Malawian women reported that virus load in breast milk collected 6 weeks after delivery was associated with perinatal transmission [14]. A smaller study of South African women ($n = 79$), which included the collection of up to 3 breast-milk samples from 1 week to 15 months after delivery, also reported that breast-milk virus levels were associated with transmission [15]. The latter study reported no significant change, over time, in breast-milk virus levels in the women from whom 2 ($n = 24$) or 3 ($n = 17$) samples were collected. Because that study did not include breast-milk samples collected earlier than 1 week after delivery (i.e., colostrum/early milk), and because the sample size and frequency of sample collection were low, a more thorough study is necessary to define the changes, over time, of breast-milk virus levels in HIV-1–infected women.

In 1992 in Nairobi, Kenya, our group initiated a randomized clinical trial of breast-feeding and formula feeding in 425 HIV-1–seropositive mothers who were followed for up to 2 years after delivery [2]. The incidence of transmission via breast milk was 16.2% (95% confidence interval [CI], 6.5%–25.9%); 44% of all transmission was attributable to breast-feeding. The majority of transmission via breast-feeding occurred early, with the infection-rate difference between the breast-feeding group and the formula-feeding group being 63% by 6 weeks after delivery, 75% by 6 months after delivery, and 87% by 12 months after delivery. As in other studies, prenatal maternal plasma virus levels, prenatal maternal CD4 T cell count, and breast-feeding were associated with mother-to-child transmission [17–20]. Up to 7 serial breast-milk samples were collected from 275 women participating in the trial, and, in a preliminary study, cell-free viral RNA and cell-associated proviral DNA were detected in breast-milk supernatant and in cell-pellet fractions, respectively [16, 21]. Because of the large number of participants and the frequency of sample collection, this trial provided a unique opportunity to investigate both the fluctuation in breast-milk virus load during the first 2 years of lactation and the association between breast-milk virus load and both transmission and maternal disease. These analyses are presented here.

SUBJECTS, MATERIALS, AND METHODS

Subjects. The subjects in this study were from a randomized clinical trial, conducted during 1992–1998 in Nairobi, Kenya, in which transmission in breast-feeding HIV-1–infected women was compared with that in formula-feeding HIV-1–infected women. The methods for enrollment, counseling, and randomization of these women have been described elsewhere [2]. HIV-1–infected women were enrolled at 32 weeks gestation, when blood samples and cervical and vaginal swabs were collected, as has been described elsewhere [22]. Breast-milk samples were collected from 1 breast/woman. In women with breast disease or mastitis, the unaffected breast was sampled [21], limiting our ability to examine the association between breast disease and virus levels. Infant blood samples were collected within the first week after delivery, at 6 weeks, 14 weeks, and 6 months after delivery, and quarterly thereafter, for up to 2 years [21]. Maternal plasma virus load, maternal CD4 and CD8 T cell counts, maternal genital HIV-1 shedding, and infant infection status had been determined in previous studies [22, 23]. None of the women in the study received antiretroviral treatment.

Analysis of virus levels in breast milk. Breast-milk samples were collected in 50-mL conical tubes and were centrifuged at 710 *g* for 20 min [16]. The lipid layer was discarded, and the clear supernatant was aspirated into a separate tube. The remaining cell pellet was stored in liquid nitrogen, for future studies. Breast milk–supernatant samples were frozen at −70°C and were shipped, either on dry ice or in liquid nitrogen, from Nairobi, Kenya, to Seattle, Washington, where they were stored at −70°C.

Breast milk–supernatant samples were thawed and were added directly to the Gen-Probe HIV-1 Viral Load Assay, as described elsewhere [24, 25]. The Gen-Probe HIV-1 Viral Load Assay is a high-throughput transcription-mediated amplification method. In brief, the assay protocol involved (1) target capture, in which magnetic beads were used to isolate HIV-1 RNA, (2) transcription-mediated amplification of HIV-1 sequences, and (3) detection of the amplified material by HIV-1–specific probes that emit fluorescence when hybridized to complementary RNA. Plasma controls for which the number of HIV-1 RNA copies was known were used to generate a standard curve allowing estimation of the concentration of HIV-1 in the starting material of the unknown samples. A 100-μL portion of breast-milk supernatant was added directly to each reaction. The number of HIV-1 RNA copies detected was multiplied by 10, to determine the number of HIV-1 RNA copies per milliliter. If the output was above the highest stan-

dard (50,000 HIV-1 RNA copies), the assay was repeated, with the initial sample volume reduced to 20 μL and with the resulting output multiplied by 50. None of the repeated assays with reduced sample volume had outputs above the highest standard. The sensitivity of the assay is >3 HIV-1 RNA copies/assay [25]. Thus, all virus levels that were ≤30 HIV-1 RNA copies/mL when 100 μL of a sample were added to the assay were considered to be undetectable.

Statistical analysis. All analyses were performed by use of SPSS (version 8.0) and viral-load data that were $\log_{10}$ transformed. For all samples in which the breast-milk virus load of HIV-1 was below the limit of detection, a value (15 HIV-1 RNA copies/mL) was assigned at the midpoint between zero and the limit of detection.

For all analyses involving transmission of HIV-1, only samples from women who reported breast-feeding their infants were utilized, including noncompliant women randomized to formula feeding. The time of transmission was considered to be the time when infection was first detected. Logistic regression analysis of breast-milk virus load and transmission included samples only up to the time of transmission and excluded samples from mothers who transmitted in utero ($n = 8$). The Cox proportional-hazards model, with breast-milk virus load as a time-dependent covariate, also included samples only up to the time of transmission and excluded samples from mothers who transmitted in utero. Linear regression, with first-breast-milk virus load (defined as the virus load obtained from the earliest breast-milk sample) grouped in $\log_{10}$ categories, was used for the analysis of the dose-response effect of breast-milk virus load and incidence of transmission of HIV-1. For analysis of breast-milk shedding, women from whom ≥2 samples were collected during any period of lactation, regardless of time of transmission, were categorized as either nonshedders (no breast-milk virus load above the threshold of detection), intermittent shedders (≥1 value above the threshold of detection and ≥1 value below it), or consistent shedders (all values above the threshold). χ^2 Test and logistic regression adjustment for maternal plasma virus load were used for analysis of shedding and transmission.

For the analyses of breast-milk HIV-1 RNA levels over time, breast-milk virus load was measured in all available breast-milk samples collected from breast-feeding women during scheduled clinical visits. The first sampling time was during the production of colostrum/early milk (defined as 0–10 days after delivery) [26]; subsequent sampling times comprised the time closest to the scheduled visit time ±1 month, with the exception of the last sampling time (the 15-month visit), which included samples collected at 14–24 months. Samples that were not collected during these sampling times were not included in the analysis ($n = 19$). If >1 sample/woman was collected during a single sampling time, the sample collected closest to the scheduled visit date was selected. The Mann-Whitney U test was used to compare breast-milk virus load in transmitting versus nontransmitting mothers. The Wilcoxon sign-rank test for paired data was used to compare breast-milk virus levels in colostrum/early milk versus those in breast milk from later sampling times.

Both for the association between breast-milk virus load of HIV-1 and either prenatal genital shedding or prenatal maternal plasma virus load of HIV-1 and for the logistic regression analysis of breast-milk virus load and transmission, only the first breast-milk sample (as defined above) was included, because this measurement was the closest temporally to the prenatal genital and plasma samples. The majority (72%) of the first breast-milk samples were colostrum/early milk; 21% were collected >10 days and <2 months after delivery, and 7% were collected ≥2 months after delivery. The correlation between breast-milk virus load and either maternal plasma virus load or maternal CD4 T cell count was analyzed by Spearman correlation coefficients. Associations between genital viral shedding and breast-milk virus load were assessed by Mann-Whitney U test and simple linear regression, and adjustments for maternal plasma virus load were performed by multivariate linear regression.

RESULTS

Subjects. A total of 648 breast-milk samples were collected from 275 women enrolled in the randomized clinical trial. Colostrum/early milk was collected from women in the breast-feeding group and from women in the formula-feeding group. Subsequent breast-milk samples were collected from women who continued to breast-feed, including women randomized to the breast-feeding group and noncompliant women randomized to the formula-feeding group; 1–7 breast-milk samples were collected from each woman (table 1). In breast-feeding women, the average duration of breast-feeding was 21.3

Table 1. Frequencies of sample number, breast-feeding, and shedding of human immunodeficiency virus type 1.

Sample number	Women tested	Breast-feeding women	Breast-feeding women in shedding category		
			None	Intermittent	Consistent
1	130 (47.3)	45	—	—	—
2	46 (16.7)	40	2	13	25
3	31 (11.3)	31	1	19	11
4	27 (9.8)	26	1	19	6
5	27 (9.8)	27	2	17	8
6	12 (4.4)	12	0	8	4
7	2 (0.7)	2	0	2	0
Total	275	183	6	78	54

NOTE. Data are no. (% of total).

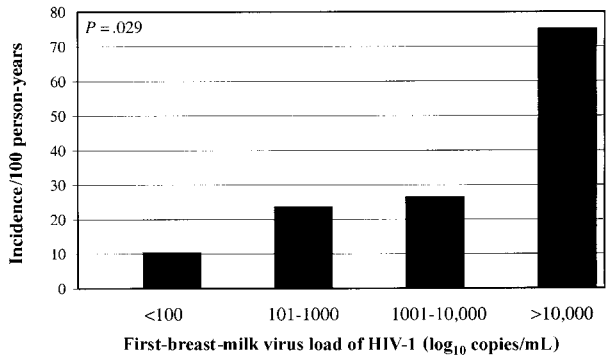

Figure 1. First-breast-milk load of human immunodeficiency virus type 1 (HIV-1)—and incidence of mother-to-child transmission.

months, ranging from <1 month to >24 months (the end of the follow-up period). The age, socioeconomic indicators, and incidence of mother-to-child transmission in the women in this study were similar to those in the entire cohort (data not shown) [2]. Of the 275 women, 70 transmitted HIV-1 to their infants whereas 205 did not; 8 women transmitted the virus in utero.

The maternal CD4 and CD8 T cell counts and maternal plasma virus load of HIV-1 in peripheral blood were available at ~32 weeks gestation [2, 23]. The mean maternal CD4 T cell count was 439 cells/μL (range, 46–1165 cells/μL). The majority (56%) of women had moderate CD4 T cell counts (range, 200–499 cells/μL), 11% had low CD4 T cell counts (<200 cells/μL), and 32.2% had high CD4 T cell counts ($\geq$500 cells/μL), on the basis of categories defined by the Centers for Disease Control and Prevention [27]. The mean maternal plasma virus load was 4.58 $\log_{10}$ HIV-1 RNA copies/mL (i.e., 37,887 copies/mL), ranging from 2.05 to 6.40 $\log_{10}$ HIV-1 RNA copies/mL (i.e., 112–2,511,886 copies/mL).

Breast-milk virus load of HIV-1. HIV-1 was detected in $\geq$1 breast-milk sample from 245 women (89%) and was below the level of detection in the remaining 30 women, from the majority (70%) of whom only 1 sample was collected. The mean breast-milk virus load in each woman was determined. The mean of these means was 2.60 $\log_{10}$ HIV-1 RNA copies/mL (i..e, 398 copies/mL), ranging from undetectable to 5.63 $\log_{10}$ HIV-1 RNA copies/mL (i.e., <30–426,580 copies/mL).

Breast-milk virus load of HIV-1 and its relation to mother-to-child transmission. The first-breast-milk virus load from each woman was a significant predictor of transmission ($P = .002$). In a Cox proportional-hazards model, each 10-fold increase in breast-milk virus load was associated with a 2.0-fold increase in risk of transmission (95% CI, 1.3–3.0; $P < .001$). A significant dose-response effect was observed between incidence of infection/100 person-years and first-breast-milk virus load ($P = .029$; see figure 1).

The maximum breast-milk virus load of HIV-1 in each woman was determined. The median maximum breast-milk virus load was significantly higher in the transmitters than in the nontransmitters (3.14 vs. 2.81 $\log_{10}$ HIV-1 RNA copies/mL; $P = .007$). The maximum breast-milk virus load also was higher (6.25 vs. 4.54 $\log_{10}$ HIV-1 RNA copies/mL). In this group, 4 women who had a breast-milk virus load >4.54 $\log_{10}$ HIV-1 RNA copies/mL transmitted HIV-1 to their infants. The minimum value (undetectable) was the same in both groups.

As a way to determine how the frequency of viral shedding affects transmission, all 138 women from whom $\geq$2 breast-milk samples were collected were classified as either nonshedders, intermittent shedders, or consistent shedders of HIV-1 RNA in their breast milk; intermittent shedders constituted the majority (56.5%) of women, followed by consistent shedders (39.1%) and nonshedders (4.3%) (table 1). The average number of samples collected from the nonshedders, the intermittent shedders, and the consistent shedders were 3.5, 3.9, and 3.2, respectively.

Of the 6 women classified as nonshedders, none transmitted the virus to their infants. Of the women who transmitted the virus to their infants, 40% were intermittent shedders and 60% were consistent shedders. Consistent shedders were significantly more likely to transmit the virus to their infant than were either intermittent shedders alone (46.3% vs. 21.7%; $P = .003$) or intermittent and nonshedders combined (46.3% vs. 20%; $P = .002$). This association remained after adjustment for maternal plasma virus load ($P = .011$).

Changes in breast-milk virus load of HIV-1 over time. The median virus load was significantly higher in colostrum/early milk than in the mature-breast-milk samples collected 14 days after delivery (2.59 vs. 2.04 $\log_{10}$ HIV-1 RNA copies/mL; $P = .004$; see table 2). For all sampling times prior to 15

Table 2. Comparison of level of human immunodeficiency virus type 1 (HIV-1) in colostrum/early milk (C/EM) to that in breast milk from later sampling times.

Sampling time(s)	Median load of HIV-1, $\log_{10}$ HIV-1 copies/mL	Comparison with C/EM	
		No. of women	*P*
C/EM (0–10 days)[a]	2.59	—	—
Visit 2 (6 weeks ± 4 weeks)	2.20	61	.09
Visit 3 (14 weeks ± 4 weeks)	2.19	53	.03
Visit 4 (6 months ± 1 month)	2.14	40	.53
Visit 5 (9 months ± 1 month)	1.48	26	.03
Visit 6 (12 months ± 1 month)	2.31	19	.40
Visit 7 (15–24 months)	2.31	11	.95
Visits 2–7 (2 weeks–24 months)	2.04[b]	80	.004

[a] A total of 105 C/EM samples from breast-feeding women were included in the analysis.
[b] Median of the means calculated for each breast-feeding woman.

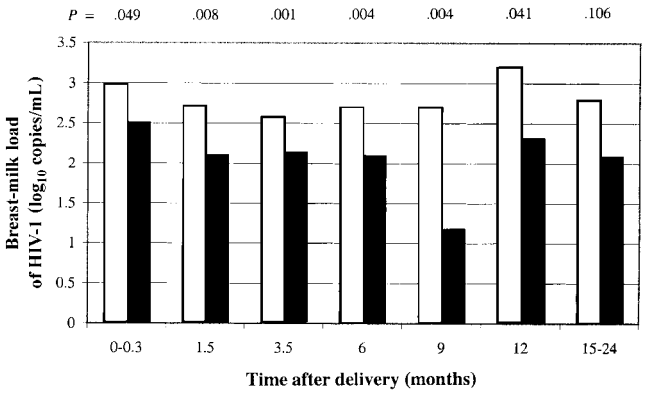

Figure 2. Breast-milk load of human immunodeficiency virus type 1 (HIV-1) over time, in all transmitting mothers *(white bars)* and nontransmitting mothers *(black bars)* who breast-fed their infants, with *P* values for comparisons of the 2 groups; for the 7 sampling times indicated, the no. of transmitting mothers was 32, 36, 31, 23, 13, 9, 6, respectively, and the no. of nontransmitting mothers was 73, 80, 74, 58, 37, 25, 13, respectively.

months, median breast-milk virus levels in transmitting mothers were significantly higher than those in nontransmitting mothers (figure 2).

Maternal correlates of breast-milk virus load of HIV-1. Maternal plasma virus load obtained at ~32 weeks gestation was correlated with first-breast-milk virus load ($r = 0.46$, $P < .001$; see figure 3*A*). Breast-milk virus load was lower, on average, than maternal plasma virus load. The median value of the ratio of maternal plasma virus load to breast-milk virus load was 1.68. For every 1-$\log_{10}$-copies/mL increase in maternal plasma virus load, there was an estimated 0.58-$\log_{10}$-copies/mL increase in breast-milk virus load ($P < .001$).

Breast-milk virus load of HIV-1 was inversely correlated with maternal CD4 T cell count ($r = -0.138$, $P = .027$; see figure 3*B*). The median breast-milk virus load in women with high ($\geq$500 cells/μL), moderate (from <500 to $\geq$200 cells/μL), and low (<200 cells/μL) CD4 T cell counts was 2.44, 2.73, and 3.19 $\log_{10}$ HIV-1 RNA copies/mL, respectively. Each 100-cells/μL decrease in CD4 T cell count was associated with a 0.113-$\log_{10}$-copies/mL increase in breast-milk virus load ($P < .001$). In a multivariate analysis that controlled for maternal plasma virus load, lower maternal CD4 T cell count remained independently associated with higher breast-milk virus load ($P = .011$).

Detection of HIV-1–infected cells in maternal cervical and vaginal secretions at ~32 weeks gestation was associated with virus load in the first breast-milk sample collected. The median breast-milk virus load was significantly higher in women whose cervical secretions contained HIV-1 DNA, compared with those whose cervical secretions did not contain it (2.91 vs. 2.52 $\log_{10}$ HIV-1 RNA copies; $P = .012$); the same was true for detection of HIV-1 DNA in maternal vaginal secretions (3.12 vs. 2.52 $\log_{10}$ HIV-1; $P = .011$). Viral shedding in maternal cervical and vaginal secretions was a significant predictor of breast-milk virus load ($P = .008$ and $P = .010$, respectively). However, these univariate relationships did not remain after adjustment for maternal plasma virus load, suggesting that there is a relationship among the virus levels in these types of maternal secretions.

DISCUSSION

This longitudinal analysis of breast-milk samples collected during the period from delivery to 2 years after delivery provides a unique and comprehensive perspective on the fluctuation in breast-milk HIV-1 levels during long-term lactation. We found that breast-milk virus levels were associated with mother-to-child transmission of HIV-1, a finding consistent with the results of 2 smaller studies [14, 15]. Specifically, we found that, throughout lactation, women who transmitted the virus to their infants had a significantly higher breast-milk virus load than did women who did not transmit the virus, and women who shed virus consistently in their breast milk were more likely to transmit HIV-1 to their infants. For each 10-fold reduction in

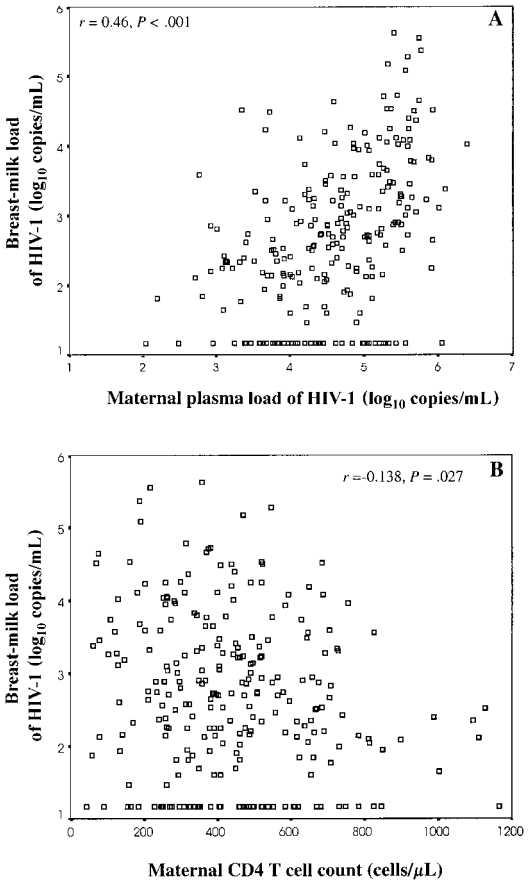

Figure 3. Correlation between breast-milk virus load in human immunodeficiency virus type 1 (HIV-1) and both maternal plasma virus load and maternal CD4 T cell count.

breast-milk virus load prior to infection, there was a 2-fold reduction in the overall rate of mother-to-child transmission. Thus, reducing breast-milk virus load may be a useful way to reduce mother-to-child transmission in settings where HIV-1–infected women breast-feed.

In contrast to what has been reported for sexual transmission [29], it was not possible to identify a breast-milk viral threshold level below which no transmission occurred. One woman in whom breast-milk virus was undetected transmitted HIV-1 to her infant, but only 1 breast-milk sample was analyzed. Also, at each visit, we measured virus from only 1 breast of each woman, and so we do not know whether virus levels were the same in both breasts. Thus, it is possible that insufficient sampling could have limited our capacity to observe a low threshold for transmission.

We found that virus levels of HIV-1 were higher in colostrum/early milk than in mature breast milk. This may explain, in part, one of the original findings of the Nairobi breast-feeding clinical trial and other studies [11, 30], which showed that the majority of breast-feeding transmission occurred during the first 6 weeks after delivery. However, infants increase their daily intake of breast milk ~1.6-fold by age 6 months [28]. Thus, infants of HIV-1–infected mothers are exposed to appreciable quantities of virus throughout lactation, despite the decline in breast-milk viral concentration over time. Breast-milk virus load may decrease over time because of declining cell concentrations in mature breast milk or, perhaps, because of the increasing effectiveness of local immune factors, some of which may reduce viral replication in the breast tissue. No significant changes in breast-milk viral RNA levels were observed in two previous studies that examined breast-milk virus load at different times during lactation, including a smaller study within this cohort [15, 16]. The significant findings in the present study most likely reflect the larger sample size and the fact that this study is unique in comparing viral RNA load at multiple times in each woman.

In the present study, breast-milk virus load of HIV-1 was associated with both maternal plasma virus load and the level of virus in maternal genital secretions; these results are not surprising, given that HIV-1 causes a systemic infection (despite some evidence for compartmentalization of viral replication [31–34]). Both maternal CD4 T cell count and maternal plasma virus load were independent predictors of breast-milk virus load. These correlates of breast-milk virus load might serve as alternative indicators of women at risk of transmission of HIV-1 to their infants via breast milk.

In summary, we found that breast-milk virus load of HIV-1 is associated with mother-to-child transmission. Our observation that this virus load is higher in colostrum/early milk than in mature breast milk may be an explanation for the high risk of infant infection early during the breast-feeding period. However, because viral shedding occurs throughout lactation and because consistent viral shedding in breast milk is associated with transmission, intervention aimed at a reduction of breast-milk virus load throughout lactation may be necessary for optimal prevention of breast-feeding transmission of HIV-1.

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