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HIV-1 persists in breast milk cells despite antiretroviral treatment to prevent mother-to-child transmission

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Abstract

Background—The effects of short-course antiretrovirals given to reduce mother-to-child transmission (MTCT) on temporal patterns of cell-associated HIV-1 RNA and DNA in breast milk are not well defined.

Methods—Women in Kenya received short-course zidovudine (ZDV), single-dose nevirapine (sdNVP), combination ZDV/sdNVP or short-course highly active antiretroviral therapy (HAART). Breast milk samples were collected two to three times weekly for 4–6 weeks. HIV-1 DNA was quantified by real-time PCR. Cell-free and cell-associated RNA levels were quantified by the Gen-Probe HIV-1 viral load assay.

Results—Cell-free HIV-1 RNA levels in breast milk were significantly suppressed by sdNVP, ZDV/sdNVP or HAART therapy compared with ZDV between day 3 and week 4 postpartum ($P \leq 0.03$). Breast milk HIV-1 DNA levels (infected cell levels) were not significantly different between treatment arms at any timepoint during the 4–6-week follow-up. At 3 weeks postpartum, when the difference in cell-free RNA levels was the greatest comparing HAART directly with ZDV ($P = 0.0001$), median $\log_{10}$ HIV-1 DNA copies per 1×10^6 cells were 2.78, 2.54, 2.69, and 2.31 in the ZDV, sdNVP, ZDV/sdNVP and HAART arms, respectively ($P = 0.23$). Cell-associated HIV-1 RNA levels were modestly suppressed in HAART versus ZDV/sdNVP during week 3 (3.37 versus 4.02, $P = 0.04$), as well as over time according to a linear mixed-effects model.

Conclusion—Cell-free and, to a lesser extent, cell-associated HIV-1 RNA levels in breast milk were suppressed by antiretroviral regimens used to prevent MTCT. However, even with HAART, there was no significant reduction in the reservoir of infected cells, which could contribute to breast milk HIV-1 transmission.

Keywords

antiretrovirals; breast milk; cell associated; HIV; vertical transmission; viral load

Introduction

The majority of HIV-1-infected women in Africa breastfeed their infants because they lack access to clean water and formula, and there is a stigma associated with formula feeding [1]. In breastfeeding populations, HIV-1 transmission via breast milk accounts for an estimated 24–44% of infant infections [2,3]. Current WHO guidelines recommend exclusive breastfeeding if replacement feeding is not ‘acceptable, feasible, affordable, sustainable and safe’ [4], which remains true for the majority of women in Africa. Designing effective interventions to reduce breast milk transmission in these settings relies on understanding the mechanisms of breast milk HIV-1 transmission and the efficacy of antiretrovirals used during breastfeeding.

Current antiretroviral prophylactic regimens used to reduce mother-to-child transmission (MTCT) in resource poor settings were not specifically designed to reduce breastfeeding transmission. These relatively simple regimens are taken during pregnancy and/or intrapartum [4] and have varied from a short course of zidovudine (ZDV) given daily during the third trimester, to a simple single dose of nevirapine (sdNVP) given to the mother at the onset of labor and to the baby postpartum [5,6]. In places with adequate infrastructure, the WHO currently recommends a combination of these regimens, and a week of postpartum combination antiretrovirals to reduce the incidence of drug resistance [4]. However, in many developing countries, sdNVP remains widely used [7].

Previous studies suggest that sdNVP may reduce transmission via breast milk [8,9]. Nevirapine (NVP) has a half-life of 60 h and can be found in maternal plasma and breast milk up to 3 weeks postpartum after a single dose taken at the onset of labor [10–12]. Single-dose NVP has been shown to effectively reduce cell-free HIV-1 RNA levels in breast milk during the early postpartum period [8], when the majority of breast milk HIV-1 transmission occurs [2]. In addition, all three drugs [ZDV, NVP, lamivudine (3TC)] used in highly active antiretroviral therapy (HAART) for first-line treatment in Africa are found at high levels in breast milk shortly after treatment [13]. Recent studies suggest that a short course of HAART, taken through 6 months of breastfeeding, is able to reduce cell-free HIV-1 RNA levels below detection for the majority of women [14] (M.H. Chung, J.N. Kiarie, B.A. Richardson, D.A. Lehman, J. Overbaugh, J. Kinuthia, *et al.*, in preparation). However, a lack of safety and efficacy data on the use of antiretrovirals during breastfeeding has precluded the WHO from recommending their use in nursing mothers who do not need treatment for their own health [4].

Most studies examining the impact of antiretrovirals on breast milk virus have focused on cell-free virus levels. However, levels of both cell-free virus (extracellular HIV-1 RNA) and infected cells (HIV-1 DNA) in breast milk correlate with transmission risk [14,15–18]. In fact, infected cell levels remain a significant correlate even after controlling for levels of cell-free virus [15], implying that infected cells may be a significant source of transmitted virus. Virus in the majority of infected breast milk cells may be latent, and the relevant source of transmitted virus is likely the viral RNA being expressed inside the cell (cell-associated HIV-1 RNA), which has not been adequately studied. The effect of antiretrovirals on all forms of HIV-1 in breast milk may provide insight to the mechanisms of breast milk transmission and the potential effectiveness of treatment interventions. In this study, we examined how antiretroviral

regimens, used for prevention of MTCT in resource-poor settings, affect levels of both cell-free and cell-associated HIV-1 in breast milk.

Methods

Study population and sample collection

Two independent randomized clinical trials were conducted at the Mathare North City Council Clinic in Nairobi, Kenya, to compare the effects of different antiretroviral regimens on viral levels in breast milk. The methods for enrollment, randomization, follow-up, and breast milk collection for both trials have been described elsewhere [8] (M.H. Chung, J.N. Kiarie, B.A. Richardson, D.A. Lehman, J. Overbaugh, J. Kinuthia, *et al.*, in preparation). In one randomized trial, designated the ‘monotherapy trial’, pregnant women were randomized to receive either 300 mg ZDV twice daily from 34 weeks gestation until labor and 300 mg every 3 h during labor until delivery, or 200 mg of NVP at the onset of labor and 2 mg/kg of NVP to the infant within 72 h of delivery. In a subsequent trial at the same location, designated the ‘combination therapy’ trial, pregnant women were randomized to receive either ZDV (300 mg twice daily) for 6 weeks prior to delivery and NVP (200 mg) during labor and NVP (2 mg/kg) to the infant after delivery, or HAART (ZDV, NVP, 3TC) for 6 weeks prior to and 6 months after delivery. In the combination therapy trial women were excluded if their CD4 cell counts at 32 weeks gestation were less than 200 or greater than 500 cells/ μ l (M.H. Chung, J.N. Kiarie, B.A. Richardson, D.A. Lehman, J. Overbaugh, J. Kinuthia, *et al.*, in preparation), whereas CD4 cell count was not an exclusion criterion in the monotherapy trial [8].

In both trials, breast milk was collected every 2–3 days for the first 4–6 weeks postpartum and maternal blood was collected at 32 weeks gestation. Different volumes of breast milk were collected in the two trials: 2–5 ml in the monotherapy trial and 5–40 ml in the combination therapy trial [8] (M.H. Chung, J.N. Kiarie, B.A. Richardson, D.A. Lehman, J. Overbaugh, J. Kinuthia, *et al.*, in preparation). Subsets of women from the original cohorts were included in this analysis. Utilizing the randomization scheme of the monotherapy trial, the first 10 women randomized in each arm were included here and provided a total of 258 breast milk cell samples. In the combination therapy trial, all women with breast milk cell samples available were included here: 16 women in the ZDV/sdNVP arm and 18 women in the HAART arm provided a total of 341 breast milk cell samples. Infant infection rates in the original cohorts are reported elsewhere [8] (M.H. Chung, J.N. Kiarie, B.A. Richardson, D.A. Lehman, J. Overbaugh, J. Kinuthia, *et al.*, in preparation). In the subset analyzed here, there were too few infant infections to compare transmission rates between treatment arms: there were no infant infections in the sdNVP or ZDV/sdNVP arms, two infants in the ZDV arm that were positive by 1 month, and one infant in the HAART arm that was positive at delivery.

Laboratory methods

CD4 cell counts were measured on blood samples collected at 32 weeks gestation, and HIV-1 RNA levels were measured in plasma from these samples using the Gen-Probe HIV-1 viral load assay (Gen-Probe Incorporated, San Diego, California, USA) as previously described [19,20]. Breast milk samples were centrifuged at 710 g for 20 min. The lipid layer was discarded, and the supernatant was separated and frozen at -70°C [8,21]. Freezing media [70% Roswell Park Memorial Institute (RPMI) medium, 20% fetal bovine serum (FBS), 10% dimethyl sulfoxide (DMSO)] was added to the cell pellet and stored in liquid nitrogen. Breast milk cell and supernatant samples were subsequently shipped in liquid nitrogen from Nairobi to Seattle, where they were stored in liquid nitrogen and at -70°C , respectively. The samples were thawed at room temperature immediately before HIV-1 testing. In samples from all treatment arms, cell-free HIV-1 RNA levels were measured from 100 μ l of breast milk supernatant using the Gen-Probe assay. The lower limit of detection is 10 copies/assay, or 100

copies/ml on the basis of 100 μ l of breast milk tested. In the ZDV/sdNVP and HAART arms, cell-associated HIV-1 RNA levels were measured from a 50 μ l portion of the cells in freezing media using the Gen-Probe assay. Concurrently, cellular DNA was extracted from a 500 μ l portion of the cell suspension using a QIAmp DNA mini kit (Qiagen, Valencia, California, USA) from samples from all treatment arms. Both HIV-1 DNA and β -actin DNA were quantified by real-time PCR as previously described [15,22]. Cell-associated HIV-1 RNA and DNA levels were normalized to the number of cells tested (number of β -actin copies). Samples with no detectable HIV-1 DNA or RNA (less than one copy or <10 copies, respectively) were set at the midpoint between zero and the lower limit of detection (0.5 and five copies, respectively) before normalizing to the number of cells tested.

Statistical methods

All analyses were intent-to-treat and were performed using Stata version 9.2 (Stata Corporation, College Station, Texas, USA). Weekly medians and distributions of $\log_{10}$ HIV-1 RNA or DNA across treatment arms were compared using the Mann–Whitney *U* test for comparison between two groups, or the Kruskal–Wallis test for comparison of more than two groups. These tests were performed after grouping samples by weekly postpartum intervals. When multiple samples were available from a woman in a particular time interval, the mean of these samples was used in this analysis. In order to account for repeated measurements per subject, we used linear mixed-effects models with exchangeable covariance to compare changes in virus levels over time in the different treatment arms. The models were multivariate with adjustments for plasma viral load and CD4 cell count at 32 weeks gestation. To ensure that our results were not biased by negative results in samples with low cell numbers, sensitivity analyses were performed for all linear mixed-effects models and Kruskal–Wallis tests by excluding undetectable samples with fewer than 10 000 cells tested.

Results

Study populations and baseline characteristics

Pregnant women were randomized to short-course ZDV, sdNVP, combined ZDV/sdNVP, or short-course HAART in two independent randomized trials [8] (M.H. Chung, J.N. Kiarie, B.A. Richardson, D.A. Lehman, J. Overbaugh, J. Kinuthia, *et al.*, in preparation). A total of eight to 15 breast milk samples per woman were collected in the first 4–6 weeks postpartum. Baseline characteristics for the women included in this analysis, which were a subset of the original cohorts, are shown in Table 1. Age, years of education, age at first sexual intercourse, and the median number of sexual partners over a lifetime was similar between the women in all four arms. However, due to differences in enrollment criteria (see Methods), median CD4 cell count at 32 weeks gestation was higher in women in the ZDV and sdNVP arms (439 and 474 cells/ μ l) compared with women in the ZDV/sdNVP and short-course HAART arms (362 and 318 cells/ μ l). In addition, the median $\log_{10}$ HIV-1 RNA levels in plasma at 32 weeks gestation was 0.27–0.55 log values lower in the ZDV/sdNVP arm compared with the other three arms (Table 1).

Cell-free HIV-1 RNA levels in breast milk

Cell-free HIV-1 RNA was detected in 38% of 679 breast milk samples tested. Median $\log_{10}$ cell-free HIV-1 RNA levels in the HAART arm were suppressed compared with the other three arms between day 3 and week 4 postpartum (Fig. 1a). This difference was the greatest during week 3 when directly comparing HAART to ZDV: median 1.70 versus 3.54 $\log_{10}$ RNA copies/ml ($P = 0.0001$, Mann–Whitney *U* test). In addition, cell-free RNA levels were suppressed by sdNVP compared with ZDV after the first 2 days and throughout week 3 postpartum ($P \leq 0.06$, Mann–Whitney *U* test). Although the median cell-free RNA levels in the ZDV/sdNVP arm were higher than in the sdNVP-alone arm, they were lower compared with the ZDV-alone arm

($P \leq 0.17$ between day 3 and week 4, Mann–Whitney U test). In addition, a multivariate linear mixed-effects model, controlling for baseline plasma viral load and CD4 cell count, showed significant suppression in all three arms (sdNVP, ZDV/sdNVP, and HAART) compared with ZDV alone ($P \leq 0.001$). The patterns of cell-free HIV-1 RNA levels over time in the four treatment arms can be seen in the lowess curves (locally weighted regression curves) in Fig. 1b. These results, for the subsets of women studied here, are similar to those published from the larger cohorts from which they were derived [8] (M.H. Chung, J.N. Kiarie, B.A. Richardson, D.A. Lehman, J. Overbaugh, J. Kinuthia, *et al.*, in preparation).

Breast milk HIV-1 DNA levels

Median HIV-1 DNA levels (or HIV-infected breast milk cell levels) did not differ significantly between treatment arms at any time during the first 6 weeks postpartum ($P \geq 0.23$, Kruskal–Wallis test, Fig. 2a). During week 3, when the largest difference in cell-free RNA levels was observed between treatment arms, median $\log_{10}$ HIV-1 DNA copies per million cells were 2.78, 2.54, 2.69, and 2.31 in the ZDV, sdNVP, ZDV/sdNVP, and HAART arms, respectively ($P = 0.23$, Kruskal–Wallis test, Fig. 2a). There was no significant difference in the pattern over time of HIV-1 DNA in breast milk between the four treatment arms when controlling for baseline plasma viral load and CD4 cell count using a linear mixed-effects model. Modeling the change over time using a linear mixed-effects model without treatment as a covariate, the model suggests there was a 0.26 log increase in breast milk HIV-1 DNA for every 10 days during the first month postpartum. This increase in the proportion of infected cells per total cells, shown in the lowess curves in Fig. 2b, is consistent with the patterns published from untreated cohorts and most likely reflects a change in cell composition of breast milk over time [14,15].

HIV-1 DNA was detected in 75% of the 599 breast milk samples tested. However, in some samples, fewer than 10 000 cells were tested for HIV-1 DNA by real-time PCR. As the average level of infected cells to total breast milk cells in untreated women is 36 infected cells per million cells (<1 : 10 000) [15], some of these samples might not have been adequate to detect HIV. In sensitivity analyses that excluded samples with undetectable HIV-1 DNA in which less than 10 000 cells were tested, results of linear mixed-effects models and Kruskal–Wallis tests were similar to those described above (data not shown).

Cell-associated HIV-1 RNA levels in breast milk

Cell-associated HIV-1 RNA levels, which provide a measure of viral transcription within the infected cell, were determined for both the ZDV/sdNVP and HAART arms. The presence of cell-associated RNA would suggest that the infected cells detected by HIV-1 DNA PCR are not all latently infected. Cell-associated HIV-1 RNA was detectable in 46% of the 347 breast milk samples tested. The difference in median $\log_{10}$ cell-associated HIV-1 RNA copies per million cells between the two arms was not statistically significant in the first and second weeks postpartum (Fig. 3a). However, during the third week postpartum, cell-associated HIV-1 RNA levels were lower in the HAART arm compared with the ZDV/sdNVP arm (3.37 versus 4.02, $P = 0.04$, Mann–Whitney U test, Fig. 3a). Although the median cell-associated RNA levels did not differ significantly after the third week postpartum, the lowess curves suggest that levels in the ZDV/sdNVP arm increase compared with HAART after the second week and through the fourth week postpartum (Fig. 3b). Similarly, in a multivariate linear mixed-effects model, the cell-associated HIV-1 RNA levels remain unchanged in the HAART arm while rising in the ZDV/sdNVP arm over time ($P < 0.01$). Sensitivity analysis of the cell-associated RNA data, both with and without undetectable samples in which less than 10 000 cells were tested, produced similar results (data not shown).

Discussion

In this study, which compared four antiretroviral regimens used to decrease MTCT of HIV-1, the use of sdNVP, ZDV/sdNVP, or HAART led to suppression of cell-free HIV-1 RNA in breast milk (Fig. 1). However, concurrently assessed levels and patterns of cell-associated HIV-1 DNA were not significantly different between treatment arms (Fig. 2). Our observation that cell-free HIV-1 RNA in breast milk was suppressed by antiretrovirals is consistent with several other studies [8,14,23,24] (M.H. Chung, J.N. Kiarie, B.A. Richardson, D.A. Lehman, J. Overbaugh, J. Kinuthia, *et al.*, in preparation), whereas there are only limited data describing the effect of antiretrovirals on cell-associated HIV-1 in breast milk [14,24,25]. The findings of this detailed study, in which levels of HIV-1 DNA were quantified at approximately 10 timepoints during a 4–6-week follow-up, reinforce findings from a study that examined breast milk at two timepoints or less and observed no effect of HAART on HIV-1 DNA [14,25]. Studies in blood also report similar findings: levels of HIV-1 DNA are not significantly reduced in the first month of treatment, and decline by only 0.5–1 log by 1 year of HAART treatment, with an estimated half-life of approximately 20 weeks [26–30]. This similar response in breast milk and blood suggests that, systemically, there are large reservoirs of HIV-1-infected cells that persist despite treatment. Importantly, a persistent reservoir of virus in breast milk, which is consumed in large quantities by breastfeeding infants, may have an impact on breast milk transmission.

HIV-1 DNA levels indicate infected cell levels, but cannot distinguish infected cells that are latently infected from those that produce virus. Our finding that HAART had little impact on HIV-1 DNA, prompted us to ask whether cell-associated RNA levels (a measure of virus expression levels) were different in women treated with short-course HAART compared with ZDV/sdNVP. During the first 2 weeks postpartum, median cell-associated HIV-1 RNA levels were similar in the ZDV/sdNVP and HAART arms ($P \geq 0.1$). During the third week postpartum, there was an increase in cell-associated RNA in women treated with ZDV/sdNVP, whereas the levels in HAART-treated women remained suppressed ($P = 0.04$, Fig. 3). In addition, a multivariate linear mixed-effects model showed that HAART suppressed cell-associated RNA relative to ZDV/sdNVP over time ($P < 0.01$). This could result from equivalent suppression by both regimens during the first 2 weeks postpartum, when NVP remains at therapeutic levels due to its long half-life [10,11], followed by an increase in cell-associated RNA in the ZDV/sdNVP arm as NVP wanes. This suggests that, in contrast to cell-associated HIV-1 DNA, there is more suppression of cell-associated HIV-1 RNA with HAART compared with ZDV/sdNVP after the first 2 weeks postpartum. In blood, even when cell-free HIV-1 RNA is suppressed below detection, cell-associated HIV-1 RNA may persist at detectable levels [26,31]. Similarly, in our study, breast milk cell-associated HIV-1 RNA was more persistent in the presence of HAART than cell-free HIV-1 RNA: cell-associated RNA remained at detectable levels in 40% of HAART-treated samples, whereas cell-free RNA was detectable only in 17% of samples (data not shown).

The study had several strengths and limitations. The strengths of the study included evaluation of three different markers of HIV-1 in breast milk, as well as assessment at up to 10 times during serial evaluation. Although this study involved women from two independent trials with different CD4 exclusion criteria, similar study procedures were utilized and women were derived from the same clinic population, resulting in similar baseline characteristics (Table 1). The similarities between the two trials allowed evaluation of four different regimens commonly used to prevent MTCT. Limitations of the study include the lack of a control arm of untreated women as a reference for normal changes in breast milk virus levels. At the time the study was conducted, it was unethical to include a no-treatment arm because evidence had shown that short-course treatments significantly reduce transmission rates [5,6]. However, the ZDV arm effectively acts as a no-treatment arm after the first 2 days postpartum because treatment ended

at delivery and ZDV has a half-life of only 1–2 h. Moreover, the overall pattern of infected cells we observed in all four treated arms is consistent with the pattern observed in a previous study of untreated women, although the sampling in that study was not as intensive as in the current study [15]: the levels of infected cells normalized to total breast milk cells increase during the first few weeks postpartum, presumably due to a change in the proportion of susceptible to total cells [15]. In contrast, when infected cell levels were normalized per milliliter, concentrations per milliliter were highest in colostrum and decreased over the first few weeks postpartum, due to a higher concentration of total cells in early breast milk compared with later milk [15]. Here, we chose to normalize on a per cell basis rather than per volume because we did not have precise volume measurements from which the cells were separated. An additional limitation to this study is the small sample sizes (10–18 women per arm, see Table 1), which could result in a lack of power to detect a significant difference in breast milk HIV-1 DNA between treatment arms. However, these sample sizes were sufficient to detect a significant difference in cell-free HIV-1 RNA, suggesting that there would have been adequate power to detect a difference in HIV-1 DNA of the same magnitude. With this small study, we cannot rule out a modest effect on the infected cell numbers, including a possible reduction in a subset of infected cells such as activated T cells, as discussed below.

The data presented here suggest there is a large reservoir of latently infected cells in breast milk that persist despite treatment, some of which express cell-associated HIV-1 RNA even while cell-free virus is reduced below the level of detection. Because reverse transcriptase inhibitors prevent new cells from being infected, the persistence of an infected cell reservoir indicates that most infected cells in breast milk do not turn over rapidly, suggesting they are either macrophages or resting T cells, which have an estimated half-life of weeks to months [32], rather than activated T cells, which turn over within days of infection [32,33]. One possible explanation for the suppression of cell-free HIV-1 RNA without suppression of HIV-1 DNA is that infected breast milk cells produce little virus, and that most cell-free virus originates outside of the breast milk compartment [34] at a site where drugs effectively suppress virus production. However, we propose an alternative model in which the infected and activated T-cell population in breast milk produces most of the cell-free virus, and this infected/activated T-cell population quickly declines in the presence of reverse transcriptase inhibitors due to cytopathic effects of the virus and normal cell turnover. This leads to a large decline in cell-free HIV-1 RNA because these cells typically express high levels of virus [35]. HIV-1 DNA is not significantly reduced because it is estimated that T cells are a minor cell population in breast milk (<5%) [36–38], whereas macrophages are the major cell population [36,39,40]. The large pool of long-lived infected macrophages and resting T cells persists in the presence of reverse transcriptase inhibitors, but does not contribute appreciably to cell-free HIV-1 RNA because these cells express low levels of virus. Thus, as shown in Fig. 4, our model suggests that cell-free HIV-1 RNA can be suppressed by reverse transcriptase inhibitors, even when there is no significant change in the reservoir of infected cells in breast milk. However, a somewhat surprising finding is that cell-associated HIV-1 RNA levels decline less dramatically than cell-free HIV-1 RNA. One explanation for this apparent discrepancy is that virus expressed in macrophages may be sequestered in intracellular compartments rather than contributing to the cell-free virus pool, as suggested by in-vitro studies [41–43]. This supports the idea that even when cell-free virus is undetectable, there may be a reservoir of cell-associated virus capable of facilitating cell-to-cell transmission.

The findings reported here are particularly important in light of studies suggesting that infected cells are a source of transmitted virus in breast milk transmission [15,17,18]. Although infected cell levels are not significantly affected by short-course HAART, cell-free, and cell-associated RNA levels may be reduced, and this may contribute to reduced breast milk transmission rates. Alternatively, HAART may decrease transmission by providing prophylaxis to breastfeeding infants in addition to reducing the levels of infectious virus in breast milk. This idea is supported

by data from multiple studies in which infant-only treatment or extended infant prophylaxis significantly reduced transmission rates [44–49]. The ability of short-course antiretroviral regimens to reduce breast milk transmission may depend upon infant prophylaxis (both directly and through passive transfer of antiretrovirals through breast milk) in addition to effects on breast milk HIV-1. These findings have implications for strategies to reduce breastfeeding transmission, which may require continued examination of the role of infant prophylaxis versus maternal treatment.

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Dara A. Lehman conducted the laboratory assays, performed statistical analysis, interpreted the data and wrote the article. Michael H. Chung implemented and helped design the study, supervised the on-site data management, and helped write the paper. Grace C. John-Stewart designed the study, obtained funding, helped to implement the study, interpret the data, and write the paper. Barbra A. Richardson advised the statistical analysis, helped to design the study and write the paper. James Kiarie implemented the study, monitored adverse events, and contributed to the study's design. John Kinuthia enrolled and examined the subjects, filled the questionnaires, and implemented the study. Julie Overbaugh supervised the laboratory testing, interpretation of data, design of the study, and writing of the paper.

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(a)

Median of mean $\log_{10}$ cell-free HIV-1 RNA/ml
breast milk over weeks postpartum

Time	ZDV $\log_{10}$ RNA/ml median (IQR)	sdNVP $\log_{10}$ RNA/ml median (IQR)	ZDV/sdNVP $\log_{10}$ RNA/ml median (IQR)	HAART $\log_{10}$ RNA/ml median (IQR)	<i>P</i> -value
0–2 days	2.30 (1.7–3.3)	2.73 (1.7–4.4)	2.83 (2.2–3.5)	2.03 (1.7–2.4)	0.38
3–7 days	2.71 (1.9–2.7)	1.70 (1.7–2.7)	1.91 (1.7–2.7)	1.70 (1.7–1.8)	0.006
Week 2	2.46 (2.0–3.3)	1.74 (1.7–2.0)	1.77 (1.7–3.3)	1.70 (1.7–1.7)	0.02
Week 3	3.54 (2.2–4.0)	1.70 (1.7–2.2)	2.21 (1.9–3.3)	1.70 (1.7–1.7)	0.001
Week 4	3.11 (1.7–3.8)	1.70 (1.7–2.5)	2.11 (1.7–2.5)	1.70 (1.7–1.7)	0.03
Week 5	3.02 (1.7–4.4)	1.70 (1.7–2.2)	1.70 (1.7–2.6)	1.70 (1.7–1.9)	0.21
> 35 days	2.76 (2.1–3.9)	1.70 (1.7–2.8)	1.70 (1.7–3.0)	1.81 (1.7–2.0)	0.09

Fig. 1. Cell-free HIV-1 RNA levels in breast milk over time postpartum: Nairobi, Kenya, 2003–2005

(a) Weekly medians and interquartile ranges of $\log_{10}$ cell-free HIV-1 RNA per milliliter. *P* values on the basis of Kruskal–Wallis test. (b) Individual data points and lowess curves for the four treatment arms: ZDV --- (× symbol), sdNVP — (square symbol), ZDV/sdNVP — (circle symbol), and HAART - - - (triangle symbol).

(a)

Time	ZDV $\log_{10}$ DNA/ 10^6 cells median (IQR)	sdNVP $\log_{10}$ DNA/ 10^6 cells median (IQR)	ZDV/sdNVP $\log_{10}$ DNA/ 10^6 cells median (IQR)	HAART $\log_{10}$ DNA/ 10^6 cells median (IQR)	P-value
0–2 days	1.50 (1.3–1.7)	1.95 (1.4–2.6)	1.50 (1.4–1.8)	1.60 (1.3–2.6)	0.84
3–7 days	1.92 (1.8–2.6)	1.88 (1.4–2.9)	1.67 (1.3–2.1)	2.22 (1.6–2.3)	0.43
Week 2	2.61 (1.7–3.3)	2.15 (2.0–2.7)	2.07 (1.4–2.6)	2.14 (1.9–2.6)	0.34
Week 3	2.78 (2.2–3.3)	2.54 (1.8–3.0)	2.69 (2.2–3.1)	2.31 (2.0–2.5)	0.23
Week 4	2.81 (2.1–3.2)	2.64 (1.7–3.2)	2.62 (2.0–3.0)	2.47 (2.1–2.6)	0.45
Week 5	2.87 (2.6–3.3)	2.34 (2.2–3.2)	2.52 (2.0–2.6)	2.45 (2.3–3.0)	0.43
> 35 days	2.96 (2.2–3.7)	2.58 (2.4–3.0)	2.68 (2.2–3.1)	2.24 (2.0–2.7)	0.23

Fig. 2. HIV-1 DNA levels in breast milk over time postpartum: Nairobi, Kenya, 2003–2005

(a) Weekly medians and interquartile ranges of $\log_{10}$ HIV-1 DNA copies per million breast milk cells. *P* values on the basis of Kruskal–Wallis test. (b) Individual data points and lowess curves for the four treatment arms: ZDV --- (x symbol), sdNVP — (square symbol), ZDV/sdNVP — (circle symbol), HAART - - (triangle symbol). BMC, breast milk cell.

Fig. 3. Cell-associated HIV-1 RNA levels in breast milk over time postpartum: Nairobi, Kenya, 2003–2005

(a) Weekly medians and interquartile ranges of $\log_{10}$ cell-associated HIV-1 RNA per million breast milk cells. *P* values on the basis of Mann–Whitney *U* test. (b) Individual data points and lowess curves for the four treatment arms: ZDV/sdNVP (—, solid line), HAART (---, long-dash dotted line). BMC, breast milk cell.

Fig. 4. A model of how treatment affects cell-free and cell-associated HIV-1 levels in breast milk
 Left panel: levels of HIV-1 DNA (infected cells) and HIV-1 RNA (cell-free virus) prior to treatment. The majority of cell-free virus is produced by activated T cells, whereas macrophages and resting T cells contribute less cell-free virus. Right panel: following treatment to prevent MTCT, where virus spread is impaired, levels of infected activated T cells decrease due to normal cell turnover of infected cells, whereas levels of infected resting T cells and macrophages are only minimally reduced. The levels of cell-free virus are significantly reduced due to the reduction of infected activated T cells.

Table 1

Baseline characteristics.

	ZDV	sdNVP	ZDV/sdNVP	HAART
Year the study was conducted	2003	2003	2005	2005
Sample size per arm tested for HIV10 DNA		10	16	18
Age, median (IQR)	25 (22–28)	24 (21–30)	24 (20–30)	26 (24–29)
Years of education completed, median (IQR)	8 (7–8)	8 (7–11)	8 (7–10)	8 (7–12)
Age at first intercourse, median (IQR)	14.5 (13–16)	16.5 (14–19)	17 (16.5–18)	16 (15–18)
Median (IQR) number of sexual partners during lifetime	4 (2–6)	3 (1–4)	3 (2–4)	3 (2–4)
Volume breast milk collected (ml)	2–5	2–5	5–40	5–40
CD4 cell count required for inclusion in study (cells/ml)	No restriction	No restriction	200–500	200–500
CD4 cell count at 32 weeks gestation, median (IQR) (cells/ml)	439 (227–473)	474 (351–550)	362 (315–435)	318 (273–421)
log ₁₀ HIV-1 plasma RNA copies/ml at 32 weeks gestation, median (IQR)	4.9 (4.47–5.56)	5.15 (3.01–5.43)	4.6 (4.28–5.15)	4.87 (4.6–4.9)

HAART, Highly active antiretroviral therapy; IQR, interquartile range; sdNVP, single-dose nevirapine; ZDV, zidovudine.