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Louise Kuhn *et al.*

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Editor's Summary

Knowing When to Wean

Breast-feeding is essential for infant survival and well-being in the low-resource settings in sub-Saharan Africa most affected by the HIV-1 epidemic. This necessitates breast-feeding by HIV-1 –infected mothers despite the 10 to 15% risk of transmitting the infection to the infant via breast milk. Concentrations of HIV-1 in breast milk influence whether the breast-fed infant will acquire infection. Kuhn and colleagues conducted a clinical trial among 958 women in Lusaka, Zambia, to evaluate the safety and efficacy of exclusive breast-feeding followed by abrupt weaning at 4 months as a strategy to prevent postnatal HIV-1 transmission and promote healthy child survival. Women were randomized to wean abruptly at 4 months or to continue breast-feeding for a duration of their own choosing and were followed with their infants from delivery to 24 months postpartum. Infants were tested at regular intervals to determine their HIV-1 status, and concentrations of HIV-1 RNA and DNA were measured in breast milk at 4 and 4.5 months. Two weeks after weaning (4.5 months), HIV-1 concentrations in breast milk were substantially higher than if breast-feeding continued. Among those continuing to breast-feed at 4.5 months, HIV-1 concentrations in milk were lowest if breast-feeding was exclusive. The boost in milk HIV-1 concentrations during weaning counteracted any advantage of shortening the duration of breast-feeding on overall postnatal HIV-1 transmission risks. Breast milk is produced in response to infant suckling. The new data demonstrate that changes in the frequency of suckling as occurs with nonexclusive breast-feeding and at the time of weaning also influence HIV-1 concentrations in breast milk. The results support continuation and possible intensification of maternal antiretroviral drug treatment over the full duration of time when any breast milk exposures are likely to occur after planned weaning.

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HIV

HIV-1 Concentrations in Human Breast Milk Before and After Weaning

Louise Kuhn,[1] Hae-Young Kim,[1] Jan Walter,[2] Donald M. Thea,[3] Moses Sinkala,[4] Mwiya Mwiya,[5] Chipepo Kankasa,[5] Don Decker,[2] Grace M. Aldrovandi[2]*

Concentrations of HIV-1 RNA and DNA in mucosal compartments influence the risk of sexual transmission and mother-to-child transmission of HIV-1. Breast milk production is physiologically regulated such that supply is a function of infant demand, but whether demand also influences HIV-1 dynamics in breast milk is unknown. We tested whether minor and major changes in feeding frequency influence breast milk viral concentrations in 958 HIV-1–infected women and their infants followed, for 24 months during a trial in Lusaka, Zambia. Women were randomized to wean abruptly at 4 months or to continue breast-feeding for a duration of their own choosing. Two weeks after breast-feeding cessation (4.5 months), HIV-1 concentrations in breast milk were substantially higher (median RNA, 2708 copies/ml; DNA, 14 copies/ml) than if breast-feeding continued (median RNA, <50 copies/ml; DNA, <1 copy/ml; $P < 0.0001$). Among those continuing breast-feeding, HIV-1 concentrations in milk were higher if breast-feeding was nonexclusive (median RNA, 293 copies/ml; DNA, 2 copies/ml; $P = 0.0006$). Elevated milk viral concentrations after stopping breast-feeding explained higher than expected rates of late postnatal HIV transmission in those who weaned early. Changes in the frequency of breast-feeding peri-weaning and with nonexclusive breast-feeding influenced milk viral concentrations. This may explain the reduced risk of HIV-1 transmission associated with exclusive breast-feeding and why early weaning does not achieve the magnitude of HIV prevention predicted by models. Our results support continuation of maternal antiretroviral drug interventions over the full duration of time when any breast milk exposures may occur after planned weaning.

INTRODUCTION

Concentrations of HIV-1 RNA and DNA in mucosal fluids strongly influence the risk of sexual and mother-to-child transmission of HIV-1 (*1*–*7*). These concentrations are related to systemic viral burden, usually measured by plasma HIV-1 RNA concentrations, and to immunosuppression measured by systemic CD4 T cell counts (*8*). Mucosal HIV-1 concentrations are markedly reduced by antiretroviral drugs, although intermittent or lower viral shedding often persists in mucosal compartments despite treatment (*9*, *10*), potentially explaining why antiretroviral interventions are not 100% effective for prevention. Both sexual and mother-to-child HIV-1 transmission are inefficient, stochastic processes with no lower or upper exposure thresholds at which transmission never or always occurs, respectively (*1*–*7*).

Human milk production is a highly orchestrated process such that milk volume and composition are dynamically regulated and vary over the course of a feeding (*11*, *12*). Infant suckling is a major regulator of milk production by stimulating production of prolactin, which supports milk production; removal of milk further stimulates more milk production (*11*, *12*). These feedback loops ensure that within a few days after delivery with regular feeding, human breasts produce almost exactly the amount of milk required by a specific infant. Whether this hormonally regulated, demand-supply process influences HIV-1 dynamics in breast milk is unknown.

We conducted a randomized clinical trial in Lusaka, Zambia, to examine the safety and efficacy of early weaning to reduce HIV-1 transmission and infant mortality (*13*). Nine hundred fifty-eight HIV-1–infected women were counseled to breast-feed for at least 4 months, at which time half were encouraged to wean abruptly and the other half encouraged to breast-feed for a duration of their own choosing. Infants were followed to 24 months with regular HIV-1 DNA polymerase chain reaction (PCR) tests to determine the timing of transmission. To ascertain compliance with early weaning, breast milk was pumped for a standard duration at around 4.5 months postpartum from all women regardless of their reported feeding practice (*14*). For women who had weaned, pumping was scheduled to occur 2 weeks after the cessation of all breast-feeding. HIV-1 RNA and DNA were quantified in milk collected at 4 and 4.5 months. The unique study design and timing of sample collection, specifically a sample after weaning, allowed us to test the hypothesis that minor and major behavioral changes in feeding frequency influence HIV-1 dynamics in human breast milk.

RESULTS

Randomized groups differed in breast milk HIV-1 concentrations at the postweaning time point

There were marked differences in concentrations of HIV-1 RNA and DNA in breast milk at 4.5 months between the two randomized groups. Among 481 women randomized to early weaning, breast milk concentrations of HIV-1 RNA [median, 388 copies/ml; interquartile range (IQR), <50 to 8624] and HIV-1 DNA (median, 6 copies/ml; IQR, <1 to 69) were significantly higher than among 475 women randomized to continue breast-feeding [RNA median, <50 copies/ml (IQR, <50 to 452); DNA median, <1 copy/ml (IQR, 0 to 5); $P < 0.0001$ and $P < 0.0001$, respectively]. This is a conservative analysis because only 60.5% of women randomized to stop breast-feeding did so by 4.5 months, thus diluting differences between the randomized groups.

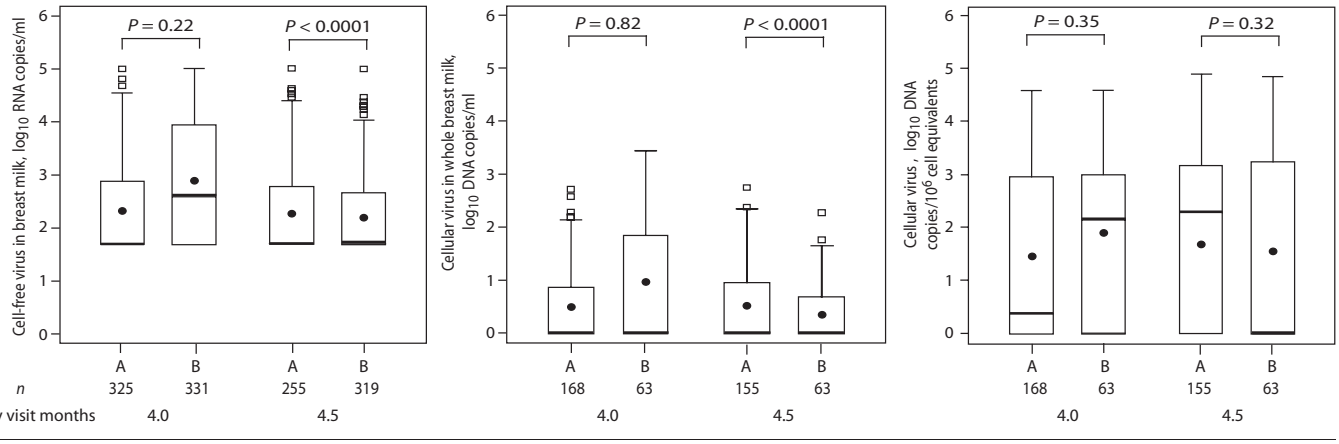
[1]Gertrude H. Sergievsky Center, and Department of Epidemiology, Mailman School of Public Health, Columbia University, New York, NY 10032, USA. [2]Department of Pediatrics, Children's Hospital Los Angeles, University of Southern California, Los Angeles, CA 90027, USA. [3]Center for International Health & Development, Boston University School of Public Health, Boston, MA 02118, USA. [4]Lusaka District Health Management Team, Lusaka, Zambia. [5]University Teaching Hospital, University of Zambia, Lusaka, Zambia.
*Corresponding author. E-mail: gracea@chla.usc.edu

This conservative, intent-to-treat analysis demonstrates that differences in viral concentrations between the groups were not due to self-selection associated with women who chose one feeding method over another. In addition, HIV-1 RNA and DNA concentrations in breast milk collected at 4 months, before any study-directed changes in feeding practices, did not differ between the groups (Fig. 1).

Women who weaned early had higher breast milk HIV-1 concentrations after weaning

To strengthen inferences about the role of changes in breast-feeding behaviors, we analyzed HIV-1 concentrations in breast milk by actual feeding behaviors. These behaviors were more heterogeneous in our study population than usually observed because of the study design. In the intervention group, 69.0% had weaned abruptly by 5 months compared to 7.4% among the controls. The median duration of breast-feeding was 4 months (IQR, 4 to 14 months) in the intervention group and 16 months (IQR, 11 to 19 months) in the control group (*13*). Breast milk HIV-1 RNA and DNA concentrations were significantly higher among those who had fully weaned by the time of milk sampling [median RNA, 2708 copies/ml (IQR, 163 to 36,790); DNA, 14 copies/ml (IQR, <1 to 81)] than among those still exclusively breast-feeding at this same age [median RNA, <50 copies/ml (IQR, <50 to 383); DNA, <1 copy/ml (IQR, <1 to 5); $P = 0.0006$ and $P < 0.0001$, respectively]. More than three-quarters (77.3%) of those who had stopped breast-feeding had breast milk RNA concentrations above the threshold of detection of 50 copies/ml [median above detection, 8166 copies/ml (IQR, 1261 to 56,568)] compared to 39.5% of those exclusively breast-feeding at this time [median above detection, 607 copies/ml (IQR, 225 to 2226); $P < 0.0001$]. These groups were not different 2 weeks earlier at 4 months (Fig. 2). These large differences in breast milk viral concentrations at 4.5 months were still observed after adjustment for potential confounders, including maternal plasma HIV-1 RNA concentrations and $CD4^+$ T cell counts.

A within-individual analysis yielded further compelling results, supporting the hypothesis that changes in feeding behaviors lead to changes in mucosal viral concentrations. The median within-woman change between 4 and 4.5 months was an increase of 1 log copy/ml (IQR, 0 to 1.78) of HIV-1 RNA in those who stopped breast-feeding, whereas in those who continued breast-feeding, there was no consistent change (median, 0 copies/ml; IQR, −0.21 to 0; $P < 0.0001$). HIV-1 DNA concentrations followed a similar pattern and were significantly elevated after weaning (Fig. 3). Among those who had undetectable HIV-1 RNA at 4 months, 58.7% had RNA concentrations above detection 2 weeks after they had stopped breast-feeding [median in those above detection, 2657 copies/ml (IQR, 1217 to 8624)]. In those who had detectable HIV-1 RNA at 4 months, the median increase was 15,973 copies/ml.



Visit months	4		4.5		4		4.5		4		4.5	
Groups	A	B	A	B	A	B	A	B	A	B	A	B
Median $\log_{10}$ HIV copies (P25, P75)	1.7 (1.7, 2.9)	1.7 (1.7, 2.8)	2.6 (1.7, 3.9)	1.7 (1.7, 2.7)	0 (0, 0.9)	0 (0, 1.0)	0.8 (0, 1.8)	0 (0, 0.7)	0.4 (0, 3.0)	2.3 (0, 3.2)	2.1 (0, 3.0)	0 (0, 3.2)
% > detection threshold	49.2	43.5	62	42.3	47.6	47.6	69.7	39.7	50.6	50.8	74.2	46
P	0.14	Ref	<0.0001	Ref	1.00	Ref	<0.0001	Ref	0.98	Ref	<0.0001	Ref
If > detection threshold median $\log_{10}$ HIV (P25, P75)	2.9 (2.3, 3.5)	2.9 (2.4, 3.6)	3.5 (2.7, 4.6)	2.8 (2.3, 3.4)	1.0 (0.5, 1.4)	1.0 (0.6, 1.6)	1.4 (0.7, 2.0)	0.9 (0.5, 1.0)	3.0 (2.5, 3.5)	3.2 (2.7, 3.8)	2.7 (1.9, 3.1)	3.4 (2.9, 3.7)

Fig. 1. Breast milk viral concentrations by randomized group. Concentrations of cell-free HIV-1 RNA, cellular HIV-1 DNA per milliliter of breast milk, and cellular HIV-1 DNA per 10^6 cell equivalents at 4 and 4.5 months from HIV-1–infected women randomized to wean at 4 months (group A) or to continue breast-feeding (group B). The numbers of samples analyzed at each visit are shown below the box plot. *P* values from Wilcoxon tests are shown for corresponding box pairs. The thick horizontal bar represents the median value, and the dot represents the mean; the bottom and top of each box represent the 25th and 75th percentiles; the lower and upper bars of each box represent the minimum values within 1.5 times the IQR. Observations beyond 1.5 times the IQR are shown as outliers. Actual values for the median and 25th and 75th percentiles (overall and only among those above detection) of cell-free HIV-1 RNA, cellular HIV-1 DNA per milliliter of breast milk, and cellular HIV-1 DNA per 10^6 cell equivalents at 4 and 4.5 months by random assignment are shown in the table. The table also shows the proportions with breast milk HIV-1 concentrations above the detection threshold defined as >50 copies/ml for HIV-1 RNA and >0 for HIV-1 DNA. χ^2 tests were used to calculate the *P* values comparing the proportions above detection across groups.

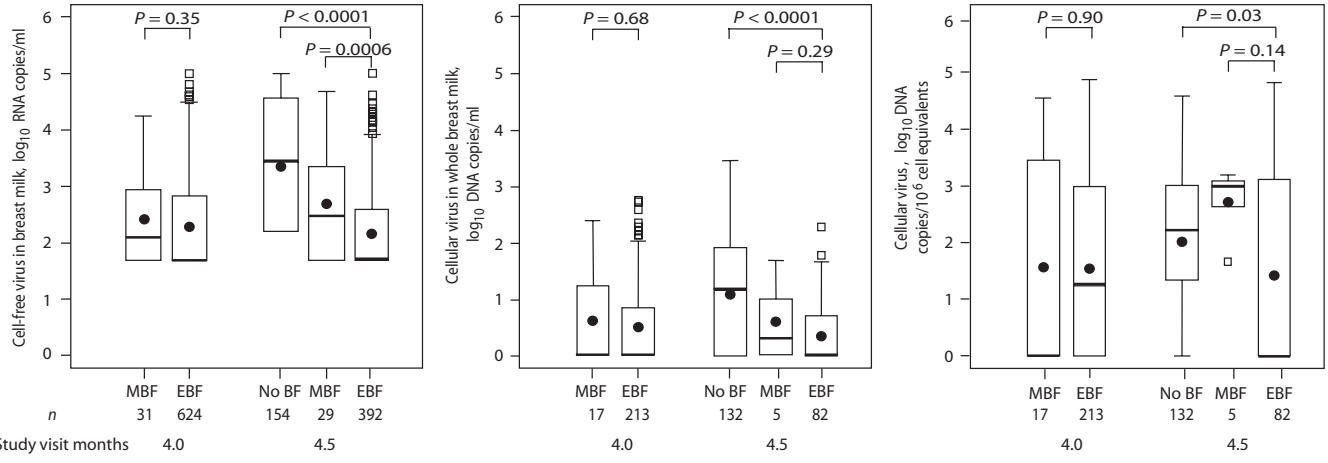
The ratio of viral RNA/DNA concentrations (in $\log_{10}$ units) increased from a median of 1.7 (IQR, 1.7 to 2.3) at 4 months before weaning to 2.2 (IQR, 1.7 to 2.7) at 4.5 months among those who weaned ($P = 0.001$). Among those still exclusively breast-feeding at 4.5 months, the median RNA/DNA ratio was 1.7 (IQR, 1.6 to 2.4) at 4 months and remained at a lower median of 1.7 (IQR, 1.7 to 2.4; $P = 0.02$) at 4.5 months.

Women practicing nonexclusive breast-feeding had higher breast milk HIV-1 concentrations than those practicing exclusive breast-feeding

Women who had given other liquids and solids to the infant in the previous 2 weeks but who were also still breast-feeding (nonexclusive or "mixed" breast-feeding) at 4.5 months had higher concentrations of breast milk HIV-1 RNA (median RNA, 293 copies/ml; IQR, <50 to 2298) at 4.5 months than those exclusively breast-feeding at this time (Fig. 2). Most women (84%) in this study had breast-fed exclusively to at least 4 months (*15*). Relative to exclusive breast-feeding, nonexclusive breast-feeding was associated with an increase of 0.52 log copy/ml [95% confidence interval (CI), 0.18 to 0.86; $P = 0.003$] at 4.5 months. Women who were nonexclusively breast-feeding at 4.5 months were doing so against study counseling advice and were more likely to be first-time mothers. There were no other significant differences between the groups. After adjustment for parity, maternal plasma HIV-1 RNA concentrations, and $CD4^+$ T cell counts (which were not collinear), there was still an association between nonexclusive breast-feeding and higher HIV-1 RNA concentrations in breast milk (0.41-log increase; 95% CI, 0.10 to 0.72). Breast milk HIV-1 RNA concentrations did not differ between women who were defined as ever versus never (before 4.5 months) mixed breast-feeders.

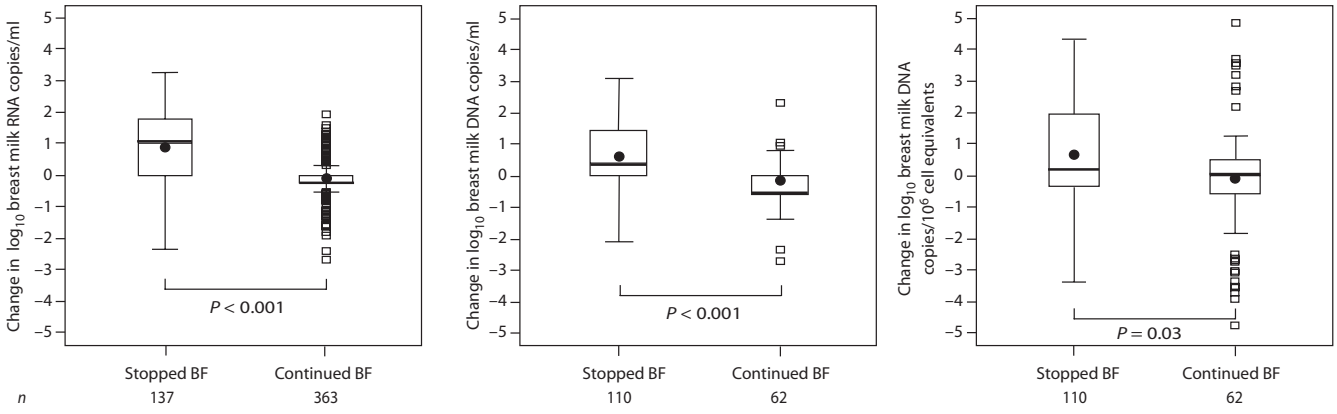
Milk volume, cellularity, and risk of breast pathology changes during weaning

Among the women who had weaned by 4.5 months, 26% produced no milk during the protocol-scheduled pumping and were thus excluded from the above analyses. Among the remaining 74% of women who had weaned, the volume of milk produced on timed pumping was significantly lower compared to volumes from women who were still breast-feeding. Weaning also led to significantly higher β-globin concentrations in milk, used as a marker of milk cellularity (Table 1). To adjust for the cellularity of the postweaning milk, we standardized HIV-1 DNA copies per million cell equivalents in breast milk. There



Visit months	4		4.5			4		4.5			4		4.5		
Feeding practice	**MBF**	**EBF**	**No BF**	**MBF**	**EBF**	**MBF**	**EBF**	**No BF**	**MBF**	**EBF**	**MBF**	**EBF**	**No BF**	**MBF**	**EBF**
Median $\log_{10}$ HIV copies (P25, P75)	2.1 (1.7, 2.9)	1.7 (1.7, 2.8)	3.4 (2.2, 4.6)	2.5 (1.7, 3.4)	1.7 (1.7, 2.6)	0 (0, 1.2)	0 (0, 0.9)	1.2 (0, 1.9)	0.3 (0, 1.0)	0 (0, 0.7)	0 (0, 3.5)	1.2 (0, 3.0)	2.2 (1.3, 3.0)	3.0 (2.6, 3.1)	0 (0, 3.1)
% > detection threshold	55	46	77.3	69	40	47.1	48	73.5	80	40.2	47.1	52	79	100	44
P	0.34	Ref	<0.0001	0.002	Ref	0.92	Ref	<0.0001	0.08	Ref	0.72	Ref	<0.0001	0.02	Ref
If > detection threshold median $\log_{10}$ HIV (P25, P75)	2.9 (2.6, 3.5)	2.9 (2.4, 3.5)	3.9 (3.1, 4.8)	3.0 (2.4, 3.8)	2.8 (2.4, 3.4)	1.3 (0.8, 1.8)	1.0 (0.6, 1.4)	1.4 (0.8, 2.1)	0.7 (0.2, 1.3)	0.8 (0.5, 1.0)	3.5 (2.8, 3.6)	3.0 (2.6, 3.5)	2.7 (1.9, 3.1)	3.0 (2.6, 3.1)	3.2 (2.8, 3.7)

Fig. 2. Breast milk viral concentrations by breast-feeding practices. Concentrations of cell-free HIV-1 RNA in copies per milliliter, cellular HIV-1 DNA in copies per milliliter of breast milk, and cellular HIV-1 DNA in copies per 10^6 cell equivalents at 4 and 4.5 months from HIV-1–infected women who had stopped breast-feeding (No BF), were mixed breast-feeding (MBF), or were exclusively breast-feeding (EBF). The numbers of samples analyzed in each group are shown below the box plot. *P* values from Wilcoxon tests are shown for corresponding box pairs. The thick horizontal bar represents the median value, and the dot represents the mean; the bottom and top of each box represent the 25th and 75th percentiles; the lower and upper bars of each box represent the minimum values within 1.5 times the IQR. Observations beyond 1.5 times the IQR are shown as outliers. Actual values for the median and 25th and 75th percentiles (overall and among those above detection) of cell-free HIV-1 RNA, cellular HIV-1 DNA per milliliter of breast milk, and cellular HIV-1 DNA per 10^6 cell equivalents in each group are shown in the table. The table also shows the proportions with breast milk HIV-1 concentrations above the detection threshold (defined as >50 copies/ml for HIV-1 RNA and >0 for HIV-1 DNA) in each group. χ^2 tests were used to calculate the *P* values comparing the proportions with breast milk HIV-1 concentrations above the detection threshold for each group relative to the EBF group.



Feeding practice	Stopped BF	Continued BF	Stopped BF	Continued BF	Stopped BF	Continued BF
Median (P25, P75)	1.00 (0, 1.78)	0.00 (−0.21, 0)	0.41 (0, 1.42)	0.00 (−0.60, 0)	0.17 (−0.37, 1.92)	0.00 (−0.58, 0.48)
Increase in $\log_{10}$ VL >1 (%)	68/137 (49.6)	16/363 (4.4)	40/110 (36.4)	2/62 (3.2)	48/110 (43.6)	12/62 (19.4)
P	<0.0001	Ref	<0.0001	Ref	0.001	Ref

Fig. 3. Change in breast milk viral concentrations between 4 and 4.5 months by breast-feeding practices. Change in concentrations of breast milk HIV-1 RNA in copies per milliliter, cellular HIV-1 DNA in copies per milliliter of breast milk, and cellular HIV-1 DNA in copies per 10^6 cell equivalents between 4 and 4.5 months stratified by whether breast-feeding (BF) had stopped or continued by 4.5 months. The numbers of paired samples analyzed in each group are shown below the box plot. *P* values from Wilcoxon tests are shown for corresponding box pairs. The thick horizontal bar represents the median value, and the dot represents the mean; the bottom and top of each box represent the 25th and 75th percentiles; the lower and upper bars of each box represent the minimum values within 1.5 times the IQR. Observations beyond 1.5 times the IQR are shown as outliers. Actual values for the median and 25th and 75th percentiles of the change in concentration between 4 and 4.5 months in each group are shown in the table. The table also shows the proportions that increased their breast milk HIV-1 concentration by more than 1 log between 4 and 4.5 months. χ^2 tests were used to calculate the *P* values comparing the proportions with a >1-log increase between those who stopped or continued breast-feeding.

Table 1. Characteristics of HIV-1–infected women at 4.5 months who had stopped or were still breast-feeding at this time. N/A, not available.

	Stopped breast-feeding	Still breast-feeding	*P*
	n = 227	*n* = 507	
n (%) on time for 4.5-month visit	197 (86.8)	432 (85.2)	
n (%) came late for pumping visit	30 (13.2)	75 (14.8)	
Median (IQR) infant age at 4.5-month visit (days)	141 (140–143)	141 (140–144)	0.61
Median (IQR) time since stopping breast-feeding (days)	14 (12–16)	N/A	
Abruptness of weaning			
Immediately	137/218 (62.8%)	N/A	
1–2 days	40/218 (18.4%)		
>3 days	41/218 (18.8%)		
No milk produced	56/215 (26.1%)	4/440 (0.9%)	<0.0001
Median (IQR) quantity of milk produced (ml)	14 (1–28)	20 (11–28)	<0.0001
Any expressed breast milk before 4.5-month study visit	183/227 (80.6%)	162/507 (32.0%)	<0.0001
Only exclusive breast-feeding before stopping or 4.5-month visit	172/227 (75.8%)	394/507 (77.7%)	0.56
Any breast problem	26/205 (12.7%)	17/503 (3.4%)	<0.0001
Maternal fever	40/208 (19.2%)	28/501 (5.6%)	<0.0001
Mastitis	18/209 (8.6%)	4/503 (0.8%)	<0.0001
	n = 132	*n* = 87	
β-Globin concentration (copies/2 μl)	7107 (842–18,023)	237 (11–774)	<0.0001

continued to be a significant increase in HIV-1 DNA copies per million cell equivalents, but the elevations were less marked than observed for concentrations of HIV-1 RNA and DNA per milliliter of breast milk (Figs. 1 and 2).

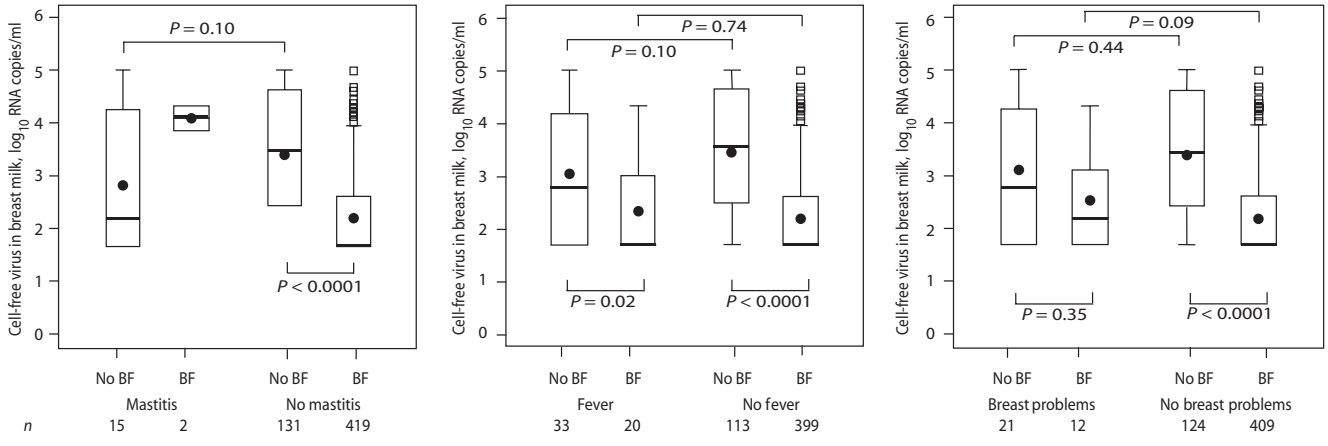
Weaning also resulted in significant increases in all breast problems ($P < 0.0001$), maternal fever ($P < 0.0001$), and clinical mastitis ($P < 0.0001$) compared to continued breast-feeding (Table 1). The increases in HIV-1 RNA and DNA in milk after weaning were independent of the increases in breast pathology and maternal fever and were most marked in women with no discernible clinical pathology (Fig. 4).

Predictors of HIV-1 concentrations in breast milk

Concentrations of HIV-1 RNA and DNA in breast milk at 4.5 months were significantly associated with maternal plasma HIV-1 concentrations, maternal $CD4^+$ T cell counts, volume of milk produced on timed pumping, and milk β-globin concentrations. The slope of the curve describing the relationship between maternal plasma HIV-1 RNA concentrations and breast milk HIV-1 RNA or DNA was almost identical whether or not breast-feeding had stopped, but the concentration of milk HIV-1 RNA was an average of 1.16 logs higher among those who had weaned. The relationship between maternal $CD4^+$ T cell counts and breast milk viral concentrations was similarly affected (Fig. 5). Breast milk HIV-1 RNA and DNA copies per milliliter were more strongly related to milk cellularity in those who had weaned than among those who were still breast-feeding (P interaction < 0.001). Pumped milk volume was only associated with breast milk HIV-1 concentrations in those who had weaned (P interaction < 0.001).

Breast milk HIV-1 RNA and DNA concentrations at 4.5 months were significantly correlated with each other in both women who were breast-feeding and those who had weaned. The relationship between DNA copies per milliliter and DNA copies per cell equivalent was somewhat weaker, although still significant, in those who had weaned compared to those who were still breast-feeding (fig. S1).

Increases in breast milk HIV-1 RNA concentrations after weaning were greater if weaning was completely abrupt (1.07; 95% CI, −1.21 to 3.35) relative to that occurring over >3 days (0.58; 95% CI, −1.71 to 2.88; $P = 0.04$). Changes in breast milk concentrations of DNA copies per milliliter were also greater if weaning was abrupt (0.85; 95% CI, −1.14 to 2.83) relative to that occurring over >1 day (0.31; 95% CI, −1.68 to 2.30; $P = 0.006$). Adherence to the study protocol that encouraged manual expression of breast milk during the weaning transition was associated with a smaller proportion of women



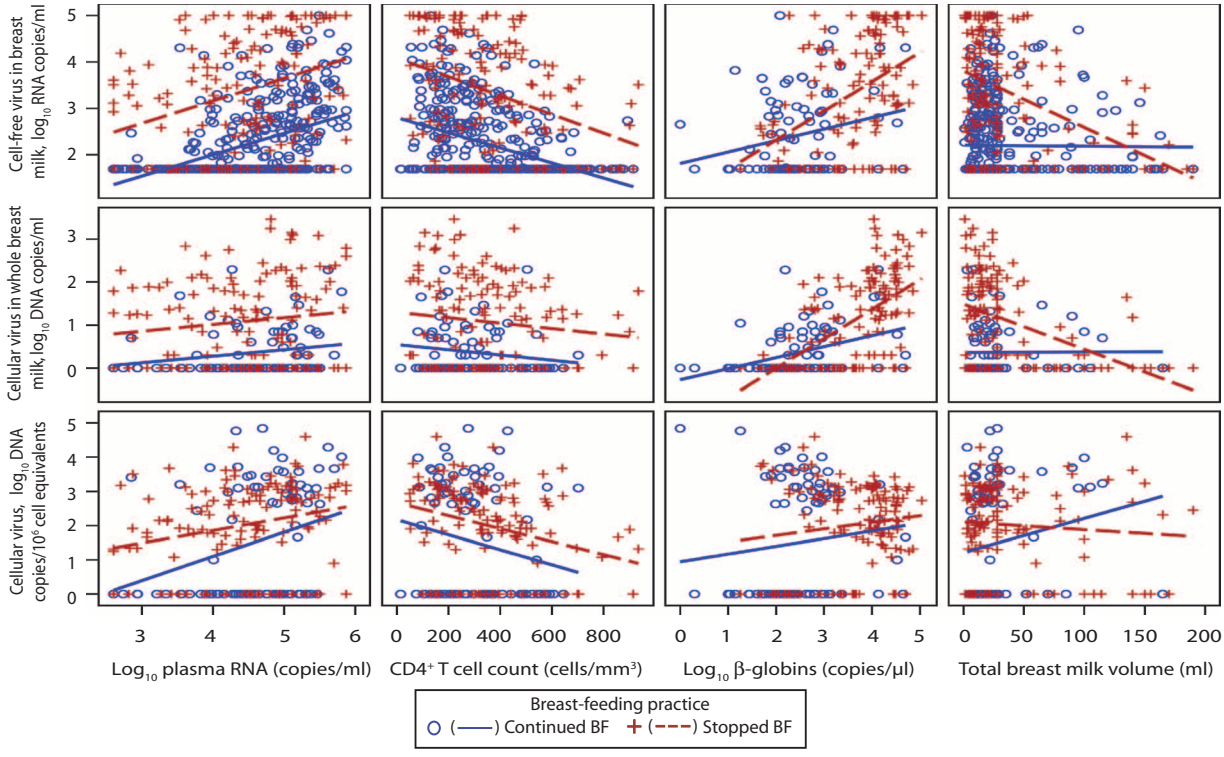
Feeding practice	Mastitis		No mastitis		Fever		No fever		Breast problems		No breast problems	
	No BF	BF	No BF	BF	No BF	BF	No BF	BF	No BF	BF	No BF	BF
Median $\log_{10}$ HIV RNA copies (P25, P75)	2.2 (1.7, 4.2)	4.1 (3.9, 4.3)	3.5 (2.4, 4.6)	1.7 (1.7, 2.6)	2.8 (1.7, 4.2)	1.7 (1.7, 3.0)	3.6 (2.5, 4.6)	1.7 (1.7, 2.6)	2.8 (1.7, 4.2)	2.2 (1.7, 3.1)	3.4 (2.4, 4.6)	1.7 (1.7, 2.6)
% >50 copies/ml	53.3	100	79.4	41.3	72.7	40	77.9	41.4	61.9	66.7	79.8	40.8
P		N/A	<0.0001	Ref	0.02	Ref	<0.0001	Ref	1.00	Ref	<0.0001	Ref
If >50 copies/ml, median $\log_{10}$ HIV RNA (P25, P75)	4.1 (2.8, 4.9)	4.1 (3.9, 4.3)	3.9 (3.1, 4.7)	2.8 (2.4, 3.4)	3.4 (2.6, 4.5)	3.3 (2.6, 4.0)	3.9 (3.2, 4.9)	2.8 (2.4, 3.3)	3.9 (3.7, 5.0)	2.7 (2.2, 3.6)	3.9 (3.1, 4.7)	2.8 (2.4, 3.4)

Fig. 4. Breast milk viral concentrations by breast-feeding practices stratified by maternal breast pathologies. Concentrations of cell-free HIV-1 RNA in breast milk at 4.5 months from HIV-1–infected women who had stopped breast-feeding (No BF) and continued breast-feeding (BF) among those who did and did not have mastitis, fever, or any breast problems at 4.5 months. The numbers of samples analyzed in each group are shown below the box plot. *P* values from Wilcoxon tests are shown for corresponding box pairs. The thick horizontal bar represents the median value, and the dot represents the mean; the bottom and top of each box represent the 25th and 75th percentiles; the lower and upper bars of each box represent the minimum values within 1.5 times the IQR. Observations beyond 1.5 times the IQR are shown as outliers. Actual values for the median and 25th and 75th percentiles (overall and only among those above detection) of cell-free HIV-1 RNA in each group are shown in the table. The table also shows the proportions with breast milk HIV-1 RNA concentrations >50 copies/ml in each group. χ^2 tests were used to calculate the *P* values comparing the proportions with breast milk HIV-1 concentrations above the detection threshold by breast-feeding status within each stratum (no mastitis, fever, no fever, breast problems, no breast problems) separately. *P* value was not calculated in the mastitis stratum because of small sample size.

(35.3%) having greater than 1-log increase in breast milk HIV-1 RNA concentrations after weaning than those who did not follow this advice (51.7%, $P = 0.02$). Whether breast-feeding had or had not been fully exclusive up to the time of weaning did not influence the magnitude of postweaning viral elevation.

Weaning-associated changes in breast milk HIV-1 concentrations affect postnatal transmission

The risk of intrauterine infection, defined as a positive PCR result within the first 3 days of life, was 5.9% ($n = 56$) in the overall cohort and rose to 12.6% ($n = 117$) when combined with intrapartum and



	Continued breast-feeding			Stopped breast-feeding		
Spearman coefficient *P*	Cell-free virus in breast milk, log_{10} RNA copies/ml	Cellular virus in whole breast milk, log_{10} DNA copies/ml	Cellular virus, log_{10} DNA copies/10^6 cell equivalents	Cell-free virus in breast milk, log_{10} RNA copies/ml	Cellular virus in whole breast milk, log_{10} DNA copies/ml	Cellular virus, log_{10} DNA copies/10^6 cell equivalents
	419	86	86	154	132	132
Plasma HIV-1 RNA (copies/ml)	0.53 <0.0001	0.22 0.04	0.28 0.01	0.36 <0.0001	0.13 0.13	0.28 0.001
	420	87	87	154	132	132
$CD4^+$ T cell count (cells/mm^3)	−0.40 <0.0001	−0.18 0.11	−0.18 0.09	−0.33 <0.0001	−0.10 0.27	−0.34 <0.0001
	86	87	87	129	132	132
β-Globins (copies/2 μl)	0.17 0.12	0.42 <0.0001	0.20 0.07	0.44 <0.0001	0.60 <0.0001	0.001 0.99
	417	87	87	153	132	132
Total breast milk volume (ml)	0.05 0.34	0.06 0.60	0.25 0.02	−0.37 <0.0001	−0.42 <0.0001	0.02 0.82

Fig. 5. Associations between breast milk viral concentrations and other milk parameters. Scatter plots showing associations between breast milk HIV-1 RNA concentrations in copies per milliliter, HIV-1 DNA concentration in copies per milliliter, and HIV-1 DNA concentration in copies per 10^6 cell equivalents and plasma HIV-1 RNA, CD4 T cell count, milk β-globin concentration, and total breast milk volume produced after timed pumping, stratified by feeding practice at 4.5 months. Spearman correlation coefficients and associated *P* values for each bivariate association in the continued breast-feeding and stopped breast-feeding groups separately as well as the number of samples analyzed for each association are shown in the table. Associations between breast milk HIV-1 RNA, β-globin, and milk volume were significantly modified by feeding practice (*P* for interactions = 0.02 and <0.0001, respectively), as were associations with breast milk HIV-1 DNA copies per milliliter (*P* for interactions = 0.0003 and 0.0008, respectively); association between breast milk HIV-1 DNA copies/10^6 cell equivalents and milk volume was significantly modified by feeding practice (*P* for interaction = 0.03). *P* values for the interactions were calculated with linear regression with multiplicative interaction terms of the relevant covariates.

very early postnatal transmission (a positive PCR result in the first 42 days of life). Almost all women breast-fed to 4 months (95.5%), and by 4 months, the transmission rate had further risen to 16.8% (n = 154). Late postnatal transmission (defined as infection detected >4 months) occurred among a further 54 infants by 24 months, yielding a total transmission rate of 24.4%.

The risk of late postnatal infection (>4 months) was 7.2% by 24 months in those who reported stopping all breast-feeding at 4 months versus 9.7% in those who were still breast-feeding at 4 months [relative hazard (RH), 0.65; 95% CI, 0.34 to 1.23]. If adjusted for the breast milk HIV-1 RNA concentration at 4.5 months, the reduction in transmission due to early weaning was stronger and became significant (RH, 0.28; 95% CI, 0.11 to 0.74). The risk of late postnatal infection in those who reported stopping was 3.5-fold higher (95% CI, 1.65 to 7.42) than expected if breast-feeding duration is taken into account by censoring follow-up time 30 days after weaning. This excess transmission risk associated with early weaning, which we observed after adjusting for breast-feeding duration, was attenuated toward the null (RH, 1.25; 95% CI, 0.42 to 3.75) once breast milk HIV-1 RNA concentrations at 4.5 months were taken into account, indicating that these breast milk viral elevations after weaning explained the excess transmission risk occurring among those who weaned early.

Viewed from an intent-to-treat perspective, the risk of late postnatal transmission was 7.6% in the group randomized to stop breast-feeding at 4 months and 10.2% in the group randomized to continue breast-feeding (RH, 0.67; 95% CI, 0.39 to 1.15). However, if adjusted for the HIV-1 RNA concentrations in breast milk collected at 4.5 months, then the benefit of being in the intervention group strengthened (RH, 0.34; 95% CI, 0.16 to 0.76). That is, if the postweaning increases in breast milk HIV concentrations were removed, early weaning would have resulted in a more than threefold decrease in the risk of late postnatal infection.

Ten of 54 (19%) late postnatal infections (that is, infections detectable only after 4 months of age in children with negative results at 4 months) occurred among infants of mothers who reported stopping all breast-feeding at 4 months. There was no change in breast milk HIV-1 RNA and DNA concentrations between 4 and 4.5 months [median change in RNA, 31 (IQR, −721 to 201); DNA, −3 (IQR, −23 to −2)] in these 10 transmissions, suggesting that these children had not stopped breast-feeding at the time the sample was taken, contrary to the maternal report. This is unlike the substantial changes observed in the group overall (described above).

Both HIV-1 RNA and DNA concentrations in breast milk were strongly associated with postnatal transmission in univariable analysis, but only HIV-1 RNA concentrations remained associated with both early and late postnatal HIV transmission after adjusting for maternal $CD4^+$ T cell counts and plasma HIV-1 RNA concentrations (which were not collinear in the model). Nonexclusive breast-feeding, breast problems, low maternal $CD4^+$ T cell counts, and a positive syphilis screening test were also independently associated with increased risk of early postnatal HIV transmission. Low maternal $CD4^+$ T cell counts and high plasma HIV-1 RNA were the only factors along with breast milk HIV-1 RNA concentrations independently associated with increased risk of late postnatal HIV transmission (Table 2).

DISCUSSION

We demonstrate that major changes in the frequency of infant feeding that occur around the time of weaning play a critical role in determining concentrations of HIV-1 in breast milk. Sudden reductions in the frequency of infant suckling associated with weaning led to more than 1-log increase in breast milk HIV-1 RNA and DNA concentrations. Both HIV-1 RNA and DNA were affected, RNA to the greatest extent. Systemic factors, including CD4 T cell counts and plasma HIV-1 RNA concentrations, continued to predict mucosal concentrations peri-weaning, but the average milk concentrations were higher than baseline. These increases in mucosal virus shedding were not explained by self-selection of women who stopped breast-feeding; differences remained strong and significant in intent-to-treat analyses, in within-person comparisons, and after adjusting for possible confounders. The amount of milk that could be produced on timed pumping, an imperfect but the most direct measurement of the frequency of milk removal (*16*), strongly and inversely correlated with concentrations of HIV-1 RNA and DNA among women who had stopped breast-feeding.

Although weaning resulted in maternal morbidity and increased risk of breast problems including mastitis (Table 1), these outcomes did not account for the elevations in milk HIV concentrations. Elevations were most marked in women without clinically evident pathologies (Fig. 4). Consistent with the elevations in breast pathologies, weaning was also associated with increases in milk cellularity, which, in turn, was strongly correlated with HIV-1 RNA and DNA concentrations in milk. However, even after standardizing HIV-1 DNA concentrations in milk for cellular content, there continued to be significantly elevated virus concentrations.

We propose that opening of the paracellular tight junctions of the mammary gland, as is known to occur during the establishment of lactation and during weaning (*16*, *17*), is the most parsimonious explanation for the findings. Animal models of lactation, as well as clinical studies in humans, have described this physiological process that occurs during weaning (*16*–*18*). Opening of the tight junctions would facilitate diffusion of cell-free as well as cell-associated virus into breast milk. This may also be associated with inflammatory processes that may serve to up-regulate viral replication in this compartment. We also cannot rule out other changes associated with weaning, such as up-regulation of milk protein synthesis, up-regulation of lactose and lipid synthesis, and increased oxidative metabolism (*18*–*21*), which may explain these elevations.

Although weaning is, by definition, a major change in the frequency of infant suckling, we also observed changes in HIV-1 concentrations in breast milk in association with more minor behavioral changes, namely, with nonexclusive breast-feeding at 4.5 months. Virus concentrations among those who were still breast-feeding but not exclusively were higher than those exclusively breast-feeding, but were lower than those who had completely stopped all breast-feeding at this time. It was not possible to determine the extent to which the other fluids and liquids given to the infant during mixed breast-feeding displaced or shortened breast milk feeds or whether they simply disrupted the regularity of breast milk feeds. Because the association was strongest at 4.5 months, we hypothesize that this was the period characterized by the most marked changes in feeding frequency and regularity. Clinical studies have found that exclusive breast-feeding during the first few months of life is the most practical method to attain a physiological balance between infant demand and milk supply to prevent "insufficient milk syndrome" and mastitis (*22*). This is one of the major reasons to support exclusive breast-feeding in addition to its benefits for infant health (*23*). Our data indicate that

Table 2. Risk factors for early (<4 months) and late (>4 months) postnatal HIV transmission among infants born to HIV-infected women in Lusaka, Zambia. Calculated with Cox proportional hazards models. Adjusted RHs are from multivariable models adjusting simultaneously for the variables shown.

	Early transmission (*n* = 37/839)		Late transmission (*n* = 54/695)*	
	RH (95% CI)		RH (95% CI)	
	Unadjusted	Adjusted	Unadjusted	Adjusted
HIV-1 concentrations in breast milk at 4 months				
HIV-1 RNA				
>500 copies/ml	19.25 (4.43–83.75)		10.32 (4.88–21.86)	
50–500 copies/ml	7.82 (1.52–40.33)		3.83 (1.60–9.13)	
<50 copies/ml	1.0		1.0	
HIV-1 RNA log copies/ml (continuous)	2.74 (1.90–3.95)	2.37 (1.54–3.66)	2.93 (2.18–3.95)	2.09 (1.49–2.95)
HIV-1 DNA				
>1 copy/ml	3.66 (1.33–10.06)		2.79 (1.29–6.04)	
Undetectable	1.0		1.0	
HIV-1 DNA log copies/ml (continuous)	2.12 (1.31–3.45)		1.52 (1.04–2.23)	
HIV-1 DNA/cell equivalents				
>1 copy/1 million cell equivalents	3.20 (1.16 – 8.81)		2.99 (1.34–6.65)	
Undetectable	1.0		1.0	
HIV-1 DNA log copies/1 million cell equivalents (continuous)	1.45 (1.09–1.93)		1.44 (1.15–1.82)	
HIV-1 concentrations in breast milk at 4.5 months				
HIV-1 RNA	N/A			
>500 copies/ml			6.56 (3.07–13.99)	
50–500 copies/ml			1.99 (0.77–5.15)	
<50 copies/ml			1.0	
HIV-1 RNA log copies/ml (continuous)			2.37 (1.74–3.23)	
HIV-1 DNA	N/A			
>1 copy/ml			2.34 (1.16–4.74)	
Undetectable			1.0	
HIV-1 DNA log copies/ml (continuous)			1.90 (1.21–2.97)	
HIV-1 DNA/cell equivalents	N/A			
>1 copy/1 million cell equivalents			2.56 (1.23–5.32)	
Undetectable			1.0	
HIV-1 DNA log copies/1 million cell equivalents (continuous)			1.32 (1.08–1.62)	
Feeding characteristics				
Nonexclusive breast-feeding†	2.90 (1.43–5.88)	3.06 (1.19–7.85)	N/A	N/A
Exclusive breast-feeding	1.0			
Any breast problems†	3.27 (1.16–9.25)		1.19 (0.16–8.81)	
Maternal factors				
$CD4^+$ T cell counts				
<350 cells/mm^3	8.21 (2.91–23.17)		5.37 (2.76–10.43)	
>350 cells/mm^3	1.0		1.0	
CD4 cell count (on continuous scale per 100 cells/mm^3)	0.52 (0.40–0.67)	0.57 (0.40–0.83)	0.59 (0.49–0.71)	0.69 (0.55–0.87)

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	Early transmission (n = 37/839)		Late transmission (n = 54/695)*	
	RH (95% CI)		RH (95% CI)	
	Unadjusted	Adjusted	Unadjusted	Adjusted
Plasma HIV-1 RNA				
>50,000 copies/ml	5.73 (2.62–12.53)		4.60 (2.53–8.38)	
<50,000 copies/ml	1.0		1.0	
Plasma HIV-1 RNA log copies/ml (continuous scale)	3.72 (2.15–6.46)		3.19 (2.05–4.96)	1.96 (1.11–3.46)
Maternal hemoglobin (g/dl)				
<10	2.16 (1.13–4.15)		1.94 (1.11–3.39)	
>10	1.0		1.0	
Rapid plasma reagin status during pregnancy‡				
Positive	2.53 (1.24–5.14)	5.00 (2.10–11.93)	0.84 (0.40–1.79)	
Negative	1.0		1.0	
Infant factors				
Birth weight (g)				
<2500	2.31 (1.01–5.28)		1.92 (0.90–4.08)	
>2500	1.0		1.0	

*Follow-up time was censored 30 days after cessation of breast-feeding for late transmission only. †Coded as time-dependent covariates. ‡A dummy variable for missing rapid plasma reagin results (screening test for syphilis) (n = 47) was included in the model.

even minor perturbations from the supply-demand dynamics can lead to an increase in breast milk HIV-1 concentration. This may partially explain the now well-established connection between exclusive breast-feeding and lower risk of postnatal HIV-1 transmission relative to nonexclusive breast-feeding (*15*, *24*–*26*).

Other studies have reported no association between nonexclusive breast-feeding and breast milk HIV-1 concentrations (*5*, *27*). However, in each of these studies, the exclusivity of breast-feeding behaviors was not linked specifically to the short time interval immediately before the breast milk HIV-1 measurement. Thus, rather than indicating conflicting results, our data suggest that the effects of nonexclusive breast-feeding on breast milk HIV-1 concentrations are closely linked in time to the disruption of full breast-feeding. That these elevations can still translate to a more than doubling of the postnatal HIV transmission rate (*15*, *24*–*26*) may seem at first surprising. However, our results are consistent with data showing increased risk of HIV transmission in association with incident HIV infection in the transmitting partner in the context of mother-to-child or sexual transmission (*28*, *29*). Together, these data suggest that sudden spikes in viral concentrations may be more important for determining transmission risk than are cumulative exposures to virus or steady-state concentrations.

Our results have profound implications for prevention of mother-to-child HIV transmission programs in settings where breast-feeding is necessary to protect infant and maternal health. Abrupt weaning is no longer officially recommended as part of infant-feeding guidance (*30*). However, our data demonstrate that even gradual weaning and more subtle changes, such as those associated with nonexclusive breast-feeding, are associated with breast milk viral elevations. In practice, it will be difficult for mothers to make small and regular enough changes in feeding frequency over a long enough period to entirely smooth out these peaks as they approach full weaning. As part of our study, we advised women to express and discard breast milk after all breast-feeding cessation as a strategy to relieve engorgement. Women who followed this advice had reduced peri-weaning breast milk viral concentrations, but the practice did not ensure that breast milk HIV-1 quantity remained at baseline levels. Although gradual reductions in breast-feeding frequency over the weeks leading up to the last planned breast milk feed should be encouraged, as should counseling about the benefits of breast milk expression, these interventions alone are unlikely to mitigate the risks of HIV transmission around this time in the absence of antiretrovirals.

We demonstrated in this study that breast milk HIV-1 RNA and DNA concentrations were strongly correlated with early and late postnatal HIV-1 transmission via breast-feeding, consistent with many other reports (*2*–*7*). In untreated, HIV-infected breast-feeding women, HIV-1 RNA above the detection threshold of most assays (>50 copies/ml) is observed in about 50% of breast milk samples with average concentrations about 2 logs lower than observed in blood (*31*). Viral shedding may be inconsistent over time and between breasts (*32*–*34*). Viral evolution studies have generated evidence both for and against compartmentalized dynamics (*35*–*37*). Viral populations in breast milk consist of a mixture of locally produced virus and diffusion of strains circulating in blood. In our analysis, after adjustment for systemic markers of HIV disease progression (CD4 T cell count and plasma HIV-1 RNA concentrations), breast milk RNA concentrations were stronger predictors of transmission than breast milk DNA. Given the strong correlation between these two parameters, as well as between mucosal and systemic viral concentrations, statistical methods have limited capability for determining the underlying biological mechanisms of transmission.

Before demonstration by several independent groups of the increased risk of infant morbidity and mortality associated with premature weaning in sub-Saharan Africa (*38*–*44*), the practice was widely recommended in many programs. Early weaning was supported as a strategy to reduce the risk of postnatal HIV transmission based on observations that there is continued risk of HIV-1 transmission throughout

the duration of breast-feeding (*45*). Reducing the duration of exposure to breast milk appears initially to be a reasonable approach to reduce HIV-1 transmission risk. Projections of the likely benefit of this behavior change were generated on the basis of estimates of transmission rates per month observed among women breast-feeding for longer durations and assuming these same transmission rates would apply when breast-feeding was of a shorter duration (*46*). Our results demonstrate the limitations of this type of extrapolation and explain why these models seriously overestimated the benefit of early weaning for HIV prevention by ignoring the more complex biological and behavioral relationships we have described here. For studies that advised early weaning, only a few continued to test infants for HIV who were no longer breast-feeding. In studies that did, some observed continued HIV transmission and attributed this to poor adherence with early weaning (*41*). Although failure to actually stop breast-feeding undoubtedly occurs, our data suggest that this may not be the prime reason why benefits of early weaning for HIV prevention are less than expected. When women attempt to wean, breast milk HIV exposures continue over the days around this period. Because HIV-1 concentrations in milk at this time are higher than usual, this period is an unusually risky one for HIV-1 transmission. In our data, the elevations in breast milk HIV-1 quantity that occurred peri-weaning entirely explained the higher than expected transmission rates in those who stopped breast-feeding early.

There are several limitations of our analysis. The study was done before antiretroviral therapy even for women with advanced disease became available in the public sector in Zambia. This allowed us to examine viral dynamics without the confounding influence of antiretrovirals. However, the dynamics of HIV in breast milk when antiretroviral drugs are given is a question in urgent need of clarification. Antiretrovirals may dampen weaning-associated breast milk elevations, making maternal treatment a more attractive preventive option than infant prophylaxis in some circumstances. Our study also could only examine weaning around 4 months of age, which was the age targeted by our study intervention. Because World Health Organization guidelines have now shifted to encourage weaning at a later age (~12 months), it is important to consider similarities and differences in weaning-associated mucosal and viral dynamics in an older child.

Weaning can only be conclusively identified in retrospect. In most practical circumstances, there is likely to be some period over which a mother reduces the frequency of breast-feeding until such time as the last breast milk exposure occurs. There are no consistent definitions of the start and end of this process, and health care workers and community members may differ in terms of their common sense understanding of these fundamental concepts. We propose that for practical purposes, the starting point of weaning be defined either from the point at which a woman is instructed to stop all breast-feeding or from the point at which she intends to stop. We recognize the subjective and context-specific limitations of this operational definition. To minimize transmission that may occur over this high-risk transition period, HIV-1–infected women should continue the antiretroviral drug interventions that they used through lactation over the full duration of time when any breast milk exposures are likely to occur. Generally, a 1- to 2-week period has been considered adequate, but this may be too short for many populations. Abrupt weaning, if it could genuinely be obtained, would be effective from an HIV transmission prevention perspective. However, weaning abruptly is associated with maternal morbidity, including mastitis, and terminating breast-feeding early is associated with increased risk of infant morbidity and mortality. Although the antiretroviral drug regimens have established efficacy over the full duration of lactation (*47*), evaluation of intensified regimens targeting the peri-weaning period may be warranted.

MATERIALS AND METHODS

Study population

The data and samples were collected from a cohort of 958 HIV-infected women recruited during pregnancy and randomized and followed with their infants prospectively from delivery to 24 months postpartum as part of the Zambia Exclusive Breastfeeding Study (ClinicalTrials.gov NCT00310726) conducted in Lusaka, Zambia (*13*). Recruitment took place between 4 May 2001 and 10 September 2004 at two primary health care clinics in Lusaka, and at that time, only single-dose nevirapine was available for prevention of mother-to-child HIV-1 transmission. Antiretroviral therapy only became available toward the end of 2004, and thus, most of the women in the cohort, even those with advanced disease, did not receive therapy. Breast milk samples collected after the initiation of antiretroviral therapy ($n = 9$) were censored. Follow-up continued through 15 December 2006. All women provided signed informed consent for participation in the study, which was approved by the Institutional Review Boards of all the investigators' institutions in the United States and Zambia.

The primary objective of the overall study was to evaluate the safety and efficacy of exclusive breast-feeding for 4 months followed by abrupt weaning as a strategy to prevent HIV transmission and promote healthy child survival. Only women who intended to breast-feed were enrolled, and all women were counseled to breast-feed exclusively to at least 4 months. Half of the cohort was individually randomized to a counseling program that encouraged early abrupt weaning at 4 months. Counseling preparation for weaning started soon after the 5-week postnatal visit and included encouragement of manual expression of milk into a cup during the period of exclusive breast-feeding to begin training the child to adjust to cup feeding. Advice for soothing a non–breast-fed child was provided, as well as education about nutrition and hygiene. Infant formula and a specially prepared fortified cereal were provided to all women in the intervention group to support weaning. Manual expression and discarding of breast milk to relieve engorgement after weaning was also encouraged. Women randomized to the control group were encouraged to continue breast-feeding exclusively to 6 months, to gradually introduce complementary foods thereafter, and to continue breast-feeding for the duration of their own choosing.

Clinical and social data were collected from women at enrollment during pregnancy. This included body mass index, clinical stage, pregnancy history, parental education, socioeconomic indicators, and household composition. $CD4^+$ cell counts (FACSCount, BD Immunocytometry Systems), hemoglobin (HemoCue system), and viral load (Roche Amplicor 1.5, Roche) were also measured at this time. Clinical data were collected at delivery and study visits, and counseling was conducted weekly in the first month, roughly every 2 weeks to 6 months, and then every 3 to 24 months. Dried blood spots were collected onto filter paper with heel sticks from infants at birth, 1 week, and 1, 2, 3, 4, 4.5, 5, 6, 9, 12, 15, 18, 21, and 24 months of age regardless of reported feeding practice. Samples were tested in batches after

follow-up was complete because infant diagnosis services were not available in Zambia at the time the study was conducted. The last available sample was tested first, and, if positive, the earliest available samples were tested sequentially until the first positive result was obtained. All positive results were confirmed on at least two separate samples; if a second sample was not available, a different spot from the same time point was tested to confirm. β-Globin was amplified from all samples to rule out false negatives on the basis of inadequate sample. Infant diagnosis was done with a validated real-time PCR for HIV-1 DNA (*48*). Feeding practices were carefully documented with a standardized questionnaire administered by a study team member not responsible for breast-feeding counseling. Exclusive breast-feeding was defined at each clinic visit as breast-feeding in the absence of all other liquids or solids with the exception of prescribed medications or vitamins since the previous visit. Nonexclusive breast-feeding at the visit would include one or more instances of provision of any liquid or solid to the child if breast-feeding was still continuing. Questions were asked at each visit about breast-feeding cessation (weaning) with questions to determine the last day at which all breast-feeding ended. Clinical outcomes in infants and women, including mortality, morbidity, and weight, were documented throughout follow-up.

All standard-of-care antenatal interventions were provided as part of the study including screening for syphilis and treatment of the women and her partner with penicillin, if necessary, malaria prophylaxis, and folate and iron supplementation. Co-trimoxazole prophylaxis for women with low CD4 counts (<200 cells/μl) was introduced during the course of the study (*49*). All standard-of-care child interventions were provided as part of the study including all vaccines, vitamin A supplementation, and growth monitoring with access to a high-energy soy protein supplement with any evidence of failure to thrive. Co-trimoxazole was provided for all infants from 6 weeks to 12 months of age.

Breast milk samples and testing

Breast milk samples were collected by manual expression from all women at 4 months postpartum. At the 4.5-month visit, scheduled to correspond to 2 weeks after the cessation of all breast-feeding for women in the intervention group, both breasts were pumped with a pulsatile electric breast pump (Medela Lactina) set at a standard rate for 10 min. Milk volume produced on timed pumping was calculated by adding the volume produced by each breast. The pumping visit could be delayed for up to 2 weeks to accommodate women who delayed their planned weaning or who missed the visit, and it was still called the 4.5-month visit. The attempt to collect milk after all breast-feeding had ended was intended as a biological marker of the extent of changes in feeding practices across the groups.

Milk was kept refrigerated at the site until transported to the local study laboratory later in the day where it was processed within 4 hours of collection. Milk was centrifuged, and the cellular portion was stored separately. The aqueous portion, including the lipid, was frozen at −80°C until later testing. HIV-1 RNA was quantified in breast milk with the Ultrasensitive Roche Amplicor Monitor assay with a lower level of detection of 50 copies/ml (Roche Molecular Systems) (*48*). HIV-1 DNA was quantified with a real-time PCR assay as previously described (*48*). β-Globin was quantified from all samples as a marker of the cellular concentration of milk. The concentration of viral DNA was quantified as copies per milliliter of breast milk as well as copies per 1 million cell equivalents with the β-globin concentration as the standard, consistent with previous studies (*3*, *50*).

All available samples collected at 4 and 4.5 months were tested for HIV-1 RNA. This included 659 samples at 4 months and 575 samples at 4.5 months. Either the left or the right breast sample was tested on the basis of random selection. This excluded maternal or infant deaths, loss to follow-up, missed visits, failure to collect the sample, and, at the 4.5-month visit, failure to produce >1 ml of milk (fig. S2). A stratified sample of 232 four-month samples and 215 four-and-a-half-month samples were tested for HIV-1 DNA. These samples were selected to include all those who had stopped breast-feeding by the 4.5-month visit, all the mothers who transmitted HIV to their infants through breast-feeding, and a random sample of all others.

Statistical methods

Breast milk RNA and DNA concentrations were normalized with $\log_{10}$ transformation. RNA concentrations below 50 copies/ml were imputed as 49 copies/ml, and DNA concentrations below detection were imputed as 1 copy/ml. Descriptive statistics, medians, IQRs displaying the 25th and 75th percentiles, and standard box plots were generated stratifying by breast-feeding group. At 4 months, we compared exclusive to mixed breast-feeding based on reported practices since the previous visit (previous month). At 4.5 months, we compared three groups: those who had stopped all breast-feeding (weaned) and those who were either exclusive or mixed breast-feeders since the previous visit (previous 2 weeks). Student's t and nonparametric Wilcoxon tests were used to test for differences across the groups for continuous variables, and Pearson's χ^2 statistics and Fisher's exact test were used for categorical variables. Spearman rank order correlation coefficients were used to describe associations between continuous variables. To test for interactions by feeding modality, we conducted linear regression with multiplicative interaction terms of relevant parameters. Risks of postnatal transmission were described with Kaplan-Meier probabilities taking time to first positive result for infections and censoring those without a positive result at their last negative PCR result. To take into account the duration of breast-feeding, follow-up time was censored 30 days after the cessation of breast-feeding in some analyses of postnatal transmission occurring after 4 months. Univariable and multivariable Cox proportional hazards models were used to describe associations between risk factors and postnatal transmission. Unadjusted and adjusted hazard ratios were reported with 95% CIs. All statistical analyses were performed with SAS (version 9.2).

SUPPLEMENTARY MATERIALS

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Fig. S1. Scatter plots showing associations between breast milk HIV-1 RNA concentrations in copies per milliliter, HIV-1 DNA concentration in copies per milliliter, and HIV-1 DNA concentration in copies per 1 million cell equivalents stratified by feeding practice at 4.5 months.

Fig. S2. Flowchart of HIV-infected women who had breast milk HIV measurements.

Breast-feeding and weaning questionnaires

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