Kinetics of Nevirapine and Its Impact on HIV-1 RNA Levels in Maternal Plasma and Breast Milk Over Time After Perinatal Single-Dose Nevirapine

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Objective: To determine kinetics after single-dose nevirapine and the impact on HIV RNA [viral load (VL)] in maternal plasma and breast milk (BM).

Methods: Cohort of 120 HIV-1–infected pregnant Ugandan women received perinatal single-dose nevirapine alone and followed up with their infants through 24 weeks postdelivery. We assessed the relationship of nevirapine concentration (tandem mass spectroscopy) and HIV-1 VL (Roche AMPLICOR HIV-1 Kit, version 1.5) in maternal plasma and BM over time.

Results: At week 1 postpartum, NVP (≥10 ng/mL) was detected in all 53 plasma and 47 of 51 (92.2%) BM samples with median (interquartile ranges) of, respectively, 171 (78–214) ng/mL and 112 (64–158) ng/mL, $P = 0.075$, which decreased subsequently with traces persisting through week 4 in plasma. Plasma and BM VL dropped by week 1 and were highly correlated at delivery ($R = 0.71$, $P < 0.001$) and week 1 ($R = 0.69$, $P < 0.001$) but not thereafter. At week 1, VL correlated inversely with NVP concentration in plasma ($R = 0.39$, $P = 0.004$) and BM ($R = 0.48$, $P = 0.013$). There was a VL rebound in both compartments, which peaked at week 4 to levels greater than those at week 1 [significantly in plasma ($P < 0.001$) but not in BM] and remained stable thereafter. Median VL was consistently greater (11- to 50-fold) in plasma than BM at all time points (all $P < 0.001$).

Conclusions: After single-dose nevirapine, NVP concentration was comparably high through week 1, accompanied by suppression of plasma and BM VL. A longer "tail" (>1 week) of potent postnatal antiretroviral drugs is warranted to minimize the observed VL rebound and potential for NVP resistance as a result of persistent NVP traces.

Key Words: nevirapine, HIV-1 RNA, maternal plasma, breast milk

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INTRODUCTION

Despite advances in prevention of mother-to-child transmission (PMTCT) of HIV-1,[1] substantial rates of postnatal transmission persist in resource-limited settings, where prolonged breastfeeding is the norm.[1,2] Indeed, breast milk (BM) transmission accounts for more than 30% of all new pediatric infections (8–12% of all exposed infants) in sub-Saharan Africa where >90% of all new perinatal infections occur.[2,3]

The World Health Organization issued rapid advice in November 2009 and more recently the guidelines to implement more complex PMTCT regimens using maternal and infant antiretroviral (ARV) strategies.[4,5] These PMTCT strategies are based on new clinical trial evidence, which showed effectiveness of both maternal and infant prophylaxis during breastfeeding.[6–8] As countries in resource-limited settings move to adopt these new World Health Organization guidelines, it is likely that the transition to the more potent but complex regimens will be slow, given that the less complex nevirapine (NVP)-containing regimens by far remain the most affordable, deliverable, and sustainable PMTCT strategies in these settings. Depending on antenatal care attendance, hospital delivery, and availability of PMTCT services at heath facilities, these NVP-based regimens usually include antenatal zidovudine, perinatal single-dose nevirapine (sdNVP) and

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zidovudine during labor and delivery, and a 1-week tail of zidovudine or zidovudine + lamivudine to minimize risk of maternal NVP resistance.[9]

The purpose of these analyses was to describe the kinetics and relationship of NVP concentration and HIV-1 RNA viral load (VL) in maternal plasma and BM after peripartum sdNVP.

METHODS

Identification of Research Participants and Follow-up

HIV-1–infected ARV naive pregnant women attending the antenatal PMTCT program, at Mulago Hospital, Kampala, Uganda, were identified and recruited by convenience sampling. Participants were enrolled from November 2003 to November 2004, and followed up through 24 weeks postdelivery. We enrolled HIV-infected women ≥18 years old, ≥28 weeks gestation, with intent to breastfeed for at least 6 weeks, able to give informed consent and willing to comply with the study requirements, living within a traceable distance, willing to be home visited, and with plans to deliver at Mulago Hospital. Exclusion criteria included partner refusal, missed sdNVP, multiple births, or involvement in HIV vaccine trials. The study was approved by Institutional Review Boards at the Uganda Virus Research Institute in Uganda and the US Centers for Disease Control and Prevention in Atlanta, GA, the sponsor organization. Written informed consent was obtained at study entry and reconfirmed after delivery.

Antenatal participants received a 200-mg tablet of NVP to take home with them and were instructed to swallow it at labor onset. Study staff recorded the date and time the tablet was taken based on maternal self-report or on actual times if observed. Study mothers were provided ongoing infant feeding counseling by study staff and encouraged to wean at 3 months consistent with the Uganda Ministry of Health infant feeding guidelines at that time, although the exact timing of breastfeeding cessation was up to the mother. Maternal demographic and clinical information including receipt of sdNVP for PMTCT, mode of delivery, infant feeding history, and AIDS-related illnesses were collected using standardized questionnaires at preentry (36 weeks gestation); delivery (day 0–3); 1, 2, 4, 6, 10, and 14 weeks; and 4-, 5-, and 6-month visits. Preentry maternal CD4 count assessment was performed at the Makerere University–Johns Hopkins University Lab, a College of American Pathologists–accredited laboratory located at the Mulago Hospital Complex. Maternal plasma for VL assessment was collected at delivery; 1, 4, and 10 weeks; and 6 months.

Colostrum and BM were collected according to a standardized protocol under the supervision of study staff. After the study, mother washed her hands with soap and water and wiped the breast with water, she manually expressed BM into a 50-mL centrifuge tube: 3–5 mL at delivery and 15–25 mL at 1, 2, 4, 6, 10, and 14 weeks, and 4-, 5-, and 6-month visits. BM samples were processed within 4 hours of collection and centrifuged at 3000 revolutions per minute for 15 minutes. This process was repeated 3–5 times to ensure complete removal of the upper lipid layer. BM supernatant was stored at −70°C until testing.

Infant clinical data were collected at birth (age 0–3 days); 1, 2, 4, 6, 10, and 14 weeks; and 6-month visits. Infant blood for polymerase chain reaction (PCR) testing was obtained at birth; 2, 6, and 14 weeks; and 6 months. If the initial PCR test was positive, a confirmatory PCR testing was done. Infants were classified as HIV infected based on 2 separate HIV-positive specimens by PCR and HIV uninfected based on at least 2 negative PCR results and no positive results during follow-up, with at least 1 PCR test at least 2 months after complete cessation of breastfeeding.

Laboratory Methods

We measured NVP concentration in a subset of randomly selected maternal plasma and whole-BM samples collected at weeks 1, 2, and 4 by high-pressure liquid chromatographic/mass spectroscopy/mass spectroscopy at Johns Hopkins University. Briefly, NVP was extracted from plasma by protein precipitation, eluted from a Zorbax Eclipse XDB-C18 column, and analyzed by tandem mass spectroscopy. Calibration standards ranged from 10 to 5000 ng/mL. The accuracy ranged from 85% to 115% with a precision of <15%. Nevirapine concentrations below the lower limit of quantification (<10 ng/mL) were considered undetectable. We measured VL on all available plasma samples using the Roche AMPLICOR Monitor test kit version 1.5 (Roche Molecular Systems Inc.; Branchburg, NJ; lower limit of detection: 400 copies per milliliter); and a subset of randomly selected BM samples using the Roche AMPLICOR Ultrasensitive Monitor test kit (lower limit of detection: 50 copies per milliliter).[10] Maternal CD4+ T-cell counts at preentry were assessed with a FACS Calibur flow cytometer (Becton Dickinson, San Jose, CA).

Statistical Methods

Medians and interquartile ranges (IQR) were used to summarize continuous data unless specified otherwise. Nevirapine concentrations <10 ng/mL were assigned a value of zero. Although no data are available that indicate the NVP concentration required for PMTCT, we used ≥100 ng/mL (10 times the in vitro IC50) based on an sdNVP prophylaxis efficacy trial that targeted achievement of NVP trough level of 100 ng/mL through week 1 postpartum.[11] Plasma VL <400 copies per milliliter were assigned a value of 399, and values >750,000 were assigned a value of 750,000. BM VL <50 copies per milliliter were assigned a value of 25. Missing values were excluded from analysis. Statistical significance for comparison of proportions was tested using Fisher exact test. Wilcoxon rank sum and Kruskal–Wallis rank sum tests were used to compare medians across groups. The differences between plasma and BM VL, and differences in VL between visits, were compared using the signed rank test, and correlations were quantified with Pearson correlation coefficient with the corresponding Mann–Whitney *U* test. Data analysis was

performed with STATA version 10.0.[12] All statistical tests were 2-sided at the 0.05 level.

RESULTS

A cohort of 120 eligible women were enrolled. The median (IQR) gestational age at enrollment was 36 weeks (34–37 weeks). After delivery, 16/120 (13.3%) mother/infant pairs were ineligible due to withdrawn consent (n = 2), missed sdNVP (n = 1), not breastfeeding (n = 4), perinatal death (n = 3), missed visits (n = 3), ARV use before 6 weeks postpartum (n = 1), and relocation (n = 2). Of the mother/infant pairs followed up after delivery, 92 (88.5%) completed 24 weeks study follow-up. Ninety-two (88.5%) and 14 (13.5%) mothers reported breastfeeding at 6 and 24 weeks, respectively.

Baseline characteristics of the study population were similar between transmitters and nontransmitters apart from maternal plasma VL, which was higher among transmitters, $P = 0.012$ (Table 1). Seventeen (16.3%) infants were HIV-1 infected through the 24 weeks follow-up; 11 of these had detectable HIV DNA at birth by PCR, 2 more at the week 2 visit, and 3 more at week 6 visit (40, 41, and 42 days old, respectively). Only 1 breastfeeding infant became infected postnatally, with undetectable HIV DNA at week 6 but positive thereafter (late transmission) at week 14 (104 days old).

NVP Concentration in Plasma and BM After Perinatal sdNVP

All the 104 mothers in follow-up after delivery self-reported taking sdNVP at labor onset. The median (IQR) duration of labor was 9 hours (7–12 hours). Almost all samples assessed for NVP had detectable concentrations in plasma (n = 53/53; 100%) and BM (n = 47/51; 92.2%) at week 1 post-sdNVP with median (IQR) of 171 ng/mL (78–214 ng/mL) and 112 ng/mL (64–158 ng/mL), $P = 0.075$, respectively. Thereafter, median NVP concentration dropped similarly in both compartments to 15 ng/mL (<10–36 ng/mL) and 15 ng/mL (<10–26 ng/mL), $P = 0.875$, respectively, at week 2, with traces of NVP persisting through week 4 in plasma but not in BM (Figs. 1A, B). At week 4, only 1/38 (2.6%) plasma sample and no BM sample assayed had detectable NVP; the observed NVP value in plasma was 19 ng/mL. NVP concentrations in BM paralleled those in plasma and were correlated at week 1 ($R = 0.577$, $P < 0.001$) and week 2 ($R = 0.383$, $P = 0.01$), but not at week 4 ($R = -0.02$, $P = 0.91$) (number of samples as in Figs. 1A, B).

Temporal Patterns of HIV-1 RNA After Perinatal sdNVP

Median concentrations of HIV-1 RNA were substantially and consistently higher (11- to 50-fold) in plasma compared with BM at each time point over 24 weeks (all $P < 0.001$). We identified direct correlations between HIV-1 RNA levels in plasma and BM in the presence of NVP at delivery ($R = 0.71$, $P < 0.001$) and week 1 ($R = 0.69$, $P < 0.001$), but not at later time points as NVP concentration waned (delivery: plasma, n = 94 and BM, n = 50; week 1: plasma, n = 93 and BM, n = 48). After sdNVP, in only 1 instance at delivery did VL in BM (2906 copies per milliliter) exceed that in plasma (894 copies per milliliter). After ingestion of sdNVP, both plasma and BM VL dropped sharply within the first week postdelivery, although the decline was not statistically significant from 18,860 (5176–57,716) copies per milliliter at delivery to 11,631 (418–8762) copies per milliliter at week 1, $P = 0.775$ in plasma, and from 316 (60–732) copies per milliliter at delivery to 25 (25–25) copies per milliliter at week 1, $P = 0.495$ in BM (Table 2). At week 1, we observed an inverse correlation between VL and NVP concentrations in plasma ($R = 0.39$, $P = 0.004$) and BM ($R = 0.48$, $P = 0.013$) (number of samples as in

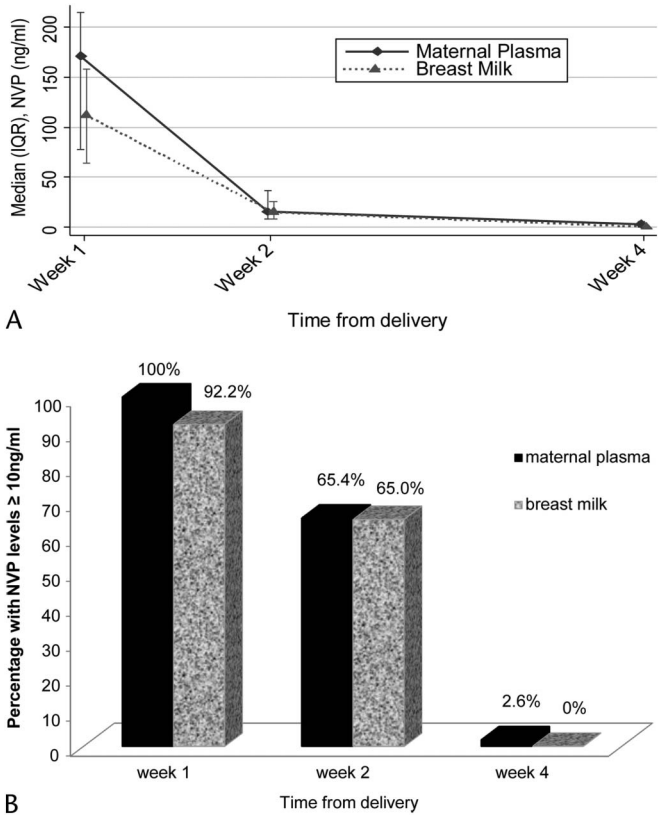

FIGURE 1. A, Center point is drawn at the median and error bars representing 25th and 75th percentiles; number of samples assayed—week 1: plasma = 53, BM = 51; week 2: plasma = 52, BM = 40; and week 4: plasma = 38, BM = 43; number of plasma samples with NVP levels <10 ng/mL assigned a value zero were 0, 18, and 37 at weeks 1, 2, and 4, respectively; number of BM samples with NVP levels <10 ng/mL assigned a value zero were 4, 14, and 43 at weeks 1, 2, and 4, respectively. B, Number of samples assayed—week 1: plasma = 53, BM = 51; week 2: plasma = 52, BM = 40; and week 4: plasma = 38, BM = 43; range of actual days when samples were collected—week 1: 6–9 days; week 2: 12–16 days; week 4: 26–34 days; number of plasma samples with NVP levels <10 ng/mL assigned a value zero were 0, 18, and 37 at weeks 1, 2, and 4, respectively; number of BM samples with NVP levels <10 ng/mL assigned a value zero were 4, 14, and 43 at weeks 1, 2, and 4, respectively.

TABLE 1. Baseline Characteristics of the Study Population by Mother-to-Child Transmission of HIV Status

Characteristic	Category	Overall (n = 120), n (%)	Nontransmitters (n = 103), n (%)	Transmitters (n = 17), n (%)	*P*
Maternal age (years)	Median (IQR)	25 (23–28)	25 (23–28)	25 (22–28)	0.721*
Gravid	Median (IQR)	3 (2–4)	3 (2–4)	2 (2–3)	0.547e*
Maternal clinical HIV stage	Stage I	94 (79.7%)	80 (79.2%)	14 (82.4%)	
	Stage II	22 (18.6%)	19 (18.8%)	3 (17.7%)	
	Stage III	2 (1.7%)	2 (2.0%)	0 (0.0)	0.833†
Caesarian section	Yes	10 (8.9%)	9 (9.3%)	1 (5.9%)	0.542†
>4 hours rupture of membranes†	Yes	108 (93.1%)	92 (85.2%)	16 (94.1%)	0.67†
Maternal CD4 cell count	Median (IQR)	422 (270–618)	429 (268–621)	384 (287–510)	0.380*
Maternal plasma HIV VL	Median (IQR)	25,799 (5780–68,712)	17,230 (3996–58,343)	57,065 (29,102–106,611)	**0.012***
Infant gender	Female	58 (51.8%)	49 (51.6%)	9 (52.9%)	0.56†
Infant birth weight (kg)	Median (IQR)	3.2 (3–3.6)	3.2 (2.9–3.6)	3.4 (3.2–3.5)	0.128*

*Wilcoxon test.
†Fisher exact test.
Bold indicates *P* value of statistical significance < 0.05.

Figs. 1A, B and Table 2, respectively). In this study, potentially protective NVP concentrations (≥100 ng/mL) were achieved in the majority of plasma (n = 35; 66%) and BM (n = 33; 64.7 %) samples at week 1. However, by week 2 only 1 (1.9%) plasma and no BM sample had NVP concentration ≥100 ng/mL.

There was a VL rebound in both compartments, which peaked at week 4 to levels greater than those at week 1, significantly in plasma ($P < 0.001$) but not in BM, and remained stable thereafter. The plasma VL at week 4 [median (IQR): 41,985 (8191–144,021) copies per milliliter] was significantly higher than that at preentry [25,799 (5780–68,712) copies per milliliter, $P = 0.012$] and at delivery [18,860 (5176–57,716) copies per milliliter, $P = 0.012$]. BM VL increased after week 1, and viral expression never returned to levels measured in the first few days after birth (Fig. 2). The ratios of plasma to BM VL were higher at the later times than at delivery and week 1 (results not shown). The apparent VL rebound in plasma after week 1 as NVP concentration diminished was observed both among mothers with high (≥10,000 copies per milliliter) or low (<10,000 copies per milliliter) preentry VL and among those with high (≥350 cells per milliliter) or low (<350 cells per milliliter) preentry CD4 cell counts.

Maternal VL showed a trend toward higher levels in plasma compared with that in BM in both the transmitters and nontransmitters (Table 2). Plasma VL (median, IQR) was significantly higher among transmitters (5- to 10-fold) compared with nontransmitters at preentry, delivery, and weeks 1, 4, 10, and 24. These differences were not as marked in BM (Table 2). In the presence of sdNVP at labor onset, the trends among transmitters and nontransmitters in both maternal plasma and BM were for VL to drop at week 1 and

TABLE 2. Maternal Plasma and BM HIV VL Through Week 24 After Perinatal sdNVP

VL	Overall, n [median (IQR)]	Nontransmitters, n [median (IQR)]	Transmitters, n [median (IQR)]	*P**
Plasma†				
Preentry	100 [25,799 (5780–68,712)]	84 [17,230 (3996–58,343)]	16 [57,065 (29,102–106,611)]	0.012
Delivery	94 [18,860 (5176–57,716)]	78 [12,465 (3825–43,306)]	16 [72,585 (35,147–164,593)]	0.004
Week 1	93 [1631 (418–8762)]	77 [1163 (399–6347)]	16 [8880 (2968–82,518)]	<0.001
Week 4	97 [41,985 (8191–144,021)]	81 [33,912 (6598–92,794)]	16 [157,749 (92,626–479,936)]	0.044
Week 10	98 [24,211 (4907–120,536)]	82 [14,977 (3995–91,138)]	16 [99,609 (16,904–200,704)]	0.001
Week 24	90 [22,171 (6502–65,745)]	74 [15,473 (5580–54,914)]	16 [98,868 (29,316–248,779)]	<0.001
BM‡				
Delivery	50 [316 (60–732)]	42 [371 (56–718)]	8 [145 (79–20,412)]	0.916
Week 1	48 [25 (25–25)]	36 [25 (25–25)]	12 [25 (25–25)]	0.278
Week 4	59 [76 (25–503)]	44 [56 (25–227)]	15 [518 (25–2914)]	0.047
Week 10	34 [77 (25–313)]	22 [58 (25–150)]	12 [279 (25–1893)]	0.04
Week 24	19 [81 (25–259)]	11 [134 (25–436)]	8 [63 (25–94)]	0.283

*Significance of difference by Kruskal–Wallis rank sum test <0.05.
†Plasma VL <400 copies per milliliter were assigned a value of 399 (n = 4 at preentry, n = 4 at delivery, n = 23 at week 1, n = 5 at week 4, n = 3 at week 10, and n = 4 at week 24), and values >750,000 were assigned a value of 750,000 (n = 2 at week 4 and n = 1 at week 10).
‡BM VL <50 copies per milliliter were assigned a value of 25 (n = 11 at delivery, n = 38 at week 1, n = 25 at week 4, n = 13 at week 10, and n = 6 at week 24).

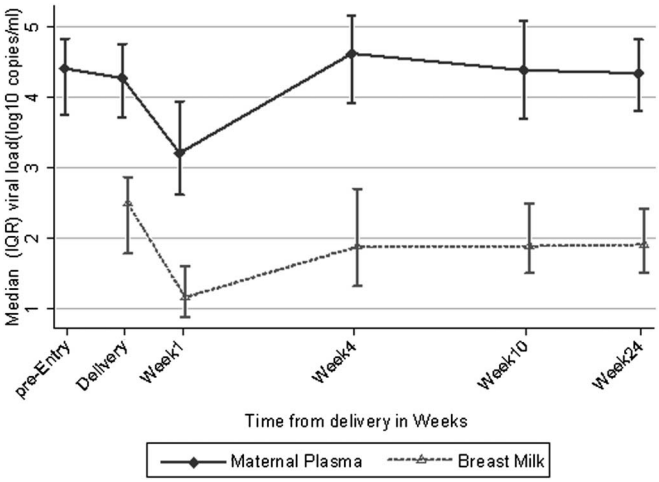

FIGURE 2. Center point is drawn at the median and error bars represent 25th and 75th percentiles.

rebound thereafter (more markedly in plasma); then return to baseline levels and remain relatively stable by weeks 10–24.

DISCUSSION

We demonstrate the kinetics of peripartum sdNVP in both maternal plasma and BM at 1, 2, and 4 weeks after sdNVP, and the impact on maternal VL in plasma and cell-free BM at delivery, and 1, 4, 10, and 24 weeks after sdNVP. We found that (*a*) effective NVP concentrations (≥100 ng/mL) were present in the majority women in plasma and BM during the early postpartum period, a time at which HIV RNA in both compartments decreased compared with perinatal values; (*b*) as NVP concentrations waned, HIV RNA rebounded in both compartments (plasma > BM) and peaked at week 4 postpartum; and (*c*) traces of NVP persisted through week 4 postpartum. This study addresses limitations of previous study designs, including cross-sectional designs, limited longitudinal time points, sample size, or available technology. The NVP concentrations from this analysis are similar to findings from previous reports based on simulation models,[13–15] with minor differences probably due to slight variations in timing of sample collection after sdNVP. A lower quantification limit (10 ng/mL, the IC_{50} of nevirapine) assay in both plasma and BM was used in this study compared with previous studies, which used 15 ng/mL in plasma and BM[13] and 50 ng/mL in plasma.[14,15]

The decline in plasma and cell-free BM VL within the first week after sdNVP supports the biological explanation for the efficacy of sdNVP in reducing early transmission of HIV from mother-to-child.[16] Indeed, we identified an inverse correlation between concentrations of NVP and VL in plasma and BM in this early postpartum period. The correlation of plasma and BM VL during this period suggests a similar NVP drug pressure in plasma and BM and underscores the potency of NVP in decreasing VL in both systemic and mucosal compartments. The observed rebound of plasma VL after the concentration of NVP wanes is consistent with recent findings[17] and similar to a VL rebound phenomenon described after discontinuation of short-course perinatal zidovudine monotherapy.[17,18]

Persistent and slowly decaying NVP concentrations may lead to selection for NVP-resistant strains of HIV-1. In a substudy of this study cohort, in which 31 (60.8%) of the 51 BM samples analyzed had successful RNA amplification, 40% of the evaluable BM samples had NVP-resistant mutations at 4 weeks after sdNVP, although these mutations faded by 6 months using standard genotypic assays.[19] Higher occurrences (65%) of BM shedding of NVP-resistant HIV-1 mutants have been reported through 8 weeks after sdNVP in a Zimbabwean cohort where the HIV-1 subtype C was predominant.[20] The VL rebound peak in this study coincided with the period of potential selection for NVP-resistant virus around week 4 after sdNVP, as described above.[19] Further studies are needed to determine whether the quasispecies comprising this VL rebound is partly populated with rapidly replicating NVP-resistant HIV-1 strains. In a setting of continued breastfeeding, this VL rebound at 1 month after sdNVP, coupled with prevalent NVP-resistant virus, could increase the risk of transmitting resistant virus strains to the infant.

Similarly, because of the relatively longer half-life of NVP, simultaneous discontinuation of an NVP-containing triple ARV regimen may result in a transient period of NVP monotherapy. Therefore, a staggered discontinuation has been suggested to minimize the risk of selecting NVP-resistant mutations.[21,22] This study was conducted before the World Health Organization guidelines in 2006 to administer additional zidovudine or zidovudine + lamivudine for 1 week after sdNVP to prevent emergence of NVP resistance.[9] Given the persistence of NVP concentrations at potentially subtherapeutic doses beyond 2 weeks in this and several other studies,[13–15] providing only a 1-week tail with zidovudine, zidovudine + lamivudine, or other nucleoside reverse transcriptase inhibitors, which have relatively shorter half-lives, may not be adequate to completely eliminate the risk of selecting for NVP-resistant strains.[23,24] A limitation of this analysis is that only about half of the plasma and BM samples collected were assayed for NVP concentration, and only about half of the BM samples were assessed for VL due to cost considerations. The subsets of assessed samples were randomly selected to minimize any potential for ascertainment bias. In addition, baseline characteristics of participants whose samples were assayed were similar to those of the general study population.

In this study, median VL after sdNVP was consistently higher in plasma than in BM, although VL in both compartments was not correlated after week 1 postpartum as NVP concentration waned. The lack of correlation contrasts with a previous study suggesting equilibrium between plasma and BM VL.[25] We did not perform cell-associated HIV assays in BM, which precludes ascertainment of the origin of the HIV within the BM compartment. However, the finding in this study of a woman with BM VL more than 3-fold the plasma VL at delivery supports studies suggesting that cell-free HIV in the BM compartment is partly produced locally by infected cells, independent of plasma VL.[26,27] Although this study was not primarily designed to assess effects on mother-to-child transmission and had limited sample size for this analysis, transmitters had 5- to 10-fold higher VL than nontransmitters

in both plasma and BM, although the differences were not significant in the latter. Indeed, maternal VL is the strongest predictor of early and late postnatal transmission[28] and the concentration of cell-free HIV-1 RNA in BM has been associated with mother-to-child transmission.[29,30]

In conclusion, after sdNVP, VL is suppressed in both plasma and BM through week 1. The VL rebound in plasma around 1 month after sdNVP, with a background of waning but low persistent NVP levels could lead to a potentially vulnerable period for (*a*) increased selection for NVP-resistant HIV-1 strains and (*b*) increased BM transmission, potentially of NVP-resistant virus. These findings warrant longer duration (>1 week) of potent postnatal ARVs including nucleoside reverse transcriptase inhibitors to eliminate the VL rebound and to further minimize the risk of NVP resistance, while assessing the risks of nucleoside reverse transcriptase inhibitor resistance virus selection.

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