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Lower Risk of Resistance After Short-Course HAART Compared With Zidovudine/Single-Dose Nevirapine Used for Prevention of HIV-1 Mother-to-Child Transmission

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Abstract

Background—Antiretroviral resistance after short-course regimens used to prevent mother-to-child transmission has consequences for later treatment. Directly comparing the prevalence of resistance after short-course regimens of highly active antiretroviral therapy (HAART) and zidovudine plus single-dose nevirapine (ZDV/sdNVP) will provide critical information when assessing the relative merits of these antiretroviral interventions.

Methods—In a clinical trial in Kenya, pregnant women were randomized to receive either ZDV/sdNVP or a short-course of HAART through 6 months of breastfeeding. Plasma samples were collected 3–12 months after treatment cessation, and resistance to reverse transcriptase inhibitors was assessed using both a sequencing assay and highly sensitive allele-specific polymerase chain reaction assays.

Results—No mutations associated with resistance were detectable by sequencing in either the ZDV/sdNVP or HAART arms at 3 months posttreatment, indicating that resistant viruses were not present in >20% of virus. Using allele-specific polymerase chain reaction assays for K103N and Y181C, we detected low levels of resistant virus in 75% of women treated with ZDV/sdNVP and only 18% of women treated with HAART ($P = 0.007$). Y181C was more prevalent than K103N at 3 months and showed little evidence of decay by 12 months.

Conclusions—Our finding provides evidence that compared with ZDV/sdNVP, HAART reduces but does not eliminate nevirapine resistance.

Keywords

antiretroviral resistance; HIV; HAART; mother-to-child transmission; prophylaxis

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INTRODUCTION

A short course of zidovudine (ZDV) from 34 weeks gestation through delivery combined with a single dose of nevirapine (sdNVP) at the onset of labor is commonly used to reduce mother-to-child transmission (MTCT) in resource-poor settings.[1] However, this and other short-course regimens that end soon after delivery do not affect breastfeeding transmission beyond the early postpartum period.[2,3] Recent studies suggest that extended prophylaxis regimens given to the infant can further reduce transmission during breastfeeding.[4] In addition, the efficacy of highly active antiretroviral therapy (HAART) taken by the mother through 6 months of breastfeeding is currently being examined in several clinical trials.[3,5–7]

Even if these extended prophylactic regimens prove efficacious, the development of resistance after their use is a concern. The emergence of resistance has not yet been examined after the extended maternal prophylactic regimens currently under consideration.[3] However, resistance has been documented to occur in HAART interruption studies[8,9] and in women following standard ZDV and sdNVP regimens used to prevent MTCT,[10,11] and evidence suggests that even low levels of resistance may reduce the efficacy of later treatment.[12–14] Resistance to nevirapine (NVP) is of particular concern because single nucleotide polymorphisms that cause amino acid changes, such as K103N and Y181C, are associated with high levels of resistance.[15,16] These mutations emerge quickly due to high viral mutation rates combined with selective pressure provided by NVP (half-life 45 hours), which can be found in plasma up to 3 weeks after a single dose.[16,17] Although combinations of antiretrovirals may reduce the incidence of NVP resistance, the addition of short-course ZDV, which ends at delivery and has a short half-life (1–2 hours), results in minimal coverage of NVP during the postpartum period. However, the addition of lamivudine (3TC), with a half-life of 5–7 hours, included in HAART (ZDV, 3TC, NVP) may provide better coverage of NVP during the period after drug cessation.

Resistance after sdNVP alone or in combination with short-course ZDV has been detected in 36% (95% confidence interval: 23% to 51%) of women 4–8 weeks postpartum.[11] These studies utilized standard sequencing assays, which can detect resistant viral variants that comprise >20% of the virus population. The use of more sensitive assays suggests that resistance can occur in up to 87% of women 4–8 weeks after sdNVP.[10,11] Few studies have examined the prevalence of resistance after short-course HAART used to prevent MTCT,[18] and none have directly compared the prevalence of resistance after short-course HAART with standard prophylactic regimens. Here we utilize both population-based sequencing and highly sensitive allele-specific polymerase chain reaction (PCR) assays to examine nonnucleoside reverse transcriptase inhibitor (NNRTI) resistance 3 months (12 weeks) after treatment. In this randomized trial, we compare the prevalence of resistance in women treated with short-course HAART with women treated with standard ZDV plus sdNVP prophylaxis.

METHODS

Study Population and Sample Collection

A randomized clinical trial was conducted in Nairobi, Kenya, with a primary outcome to compare the effect of 2 antiretroviral regimens on HIV-1 levels in breast milk. The institutional review boards at the University of Washington, United States, and Kenyatta National Hospital, Kenya, approved the study. The ClinicalTrials.gov identifier was NCT00167674. The methods for enrollment, randomization, follow-up, and sample collection have been described elsewhere.[19,20] Briefly, pregnant women were screened at 32 weeks gestation, blood was collected, and CD4 count was determined. If the CD4 count was <200 cells per cubic millimeter or >500 cells per cubic millimeter, the subject was ineligible for randomization and was referred to a treatment center for free HAART or to the antenatal clinic for standard HIV-1 perinatal care, respectively, according to the World Health Organization guidelines at the time the study

was conducted, as previously described.[19] Eligible women were randomized at 34 weeks gestation to receive either (1) ZDV (300 mg twice daily) for 6 weeks before delivery plus NVP (200 mg) during labor and NVP (2 mg/kg) to the infant after delivery or (2) HAART (300-mg ZDV, 200-mg NVP, 150-mg 3TC) twice daily for 6 weeks before and 6 months after delivery.

During follow-up, maternal blood was collected at delivery, 2 weeks, and 1, 3, 6, 9, and 12 months postpartum. Blood samples were centrifuged to separate plasma from peripheral blood mononuclear cells. Plasma samples were frozen and shipped to Seattle, WA, in liquid nitrogen and stored at −80°C until use. HIV-1 RNA levels were measured in plasma by the Gen-Probe HIV-1 viral load assay (Gen-Probe Incorporated, San Diego, CA).[21,22] Subsequently, viral RNA was extracted from a second aliquot of 140 μL from the same plasma sample using a Qiamp viral RNA kit (Qiagen, Valencia, CA) according to the manufacturer's instructions.

Construction of Wild Type, K103N, and Y181C RNA Standards

A portion of the HIV-1 *pol* gene was subcloned from full-length proviral clones of subtype A (pQ1123, accession# AF004885) and subtype D (p2059, accession# AF133821) into PCR4-TOPO (Invitrogen, Carlsbad, CA). Subsequently, site-directed mutagenesis was performed to independently introduce K103N (AAC) and Y181C (TGT). Both the wild-type and mutant HIV-1 *pol* plasmids were purified (Qiagen miniprep kit, Valencia, CA) and linearized, followed by T7 or T3 transcription using the Ambion Megascript kit (Ambion; Austin, TX). Wild type, K103N, or Y181C RNA standards were quantified by ultraviolet spectroscopy diluted to 10^4 copies per microliter in carrier RNA and stored at −80°C until use.

Population-Based Sequencing to Detect Drug Resistance

A 645 base pair region of HIV-1 *pol* was amplified using nested reverse transcriptase–polymerase chain reaction (RT-PCR). First round RT-PCR was performed with the SuperScript III one-step RT-PCR kit (Invitrogen) in a volume of 25 μL with final concentrations of 4 ng/μL each of primers RT18 (5′-GGAAACCAAAAATGATAGGGGGAATTGGAGG-3′) and RT21 (5′-CTGTATTTCTGCTATTAAGTCTTTTGATGG-3′),[23] 1x reaction mix to which 0.5 μL SuperScript III RT/Platinum *Taq* mix and 2 μL of either extracted viral RNA, no-template controls, or mixtures of mutant and wild-type RNA transcripts were added. Thermocycling conditions were 45°C for 60 minutes; 95°C for 2 minutes; 35 cycles of 95°C for 30 seconds, 55°C for 30 seconds, and 72°C for 2 minutes, followed by 72°C for 10 minutes.

Subsequently, a second-round PCR was performed in 50 μL with final reagent concentrations of 5 ng/μL primers RT1 (5′-CCAAAAGTTAAACAATGGCCATTGACAGA-3′) and RT4 (5′-AGTTCATAACCCATCCAAAG-3′),[23] 2.5 mM magnesium chloride, 0.2 mM each deoxynucleoside triphosphate, 1x AmpliTaq buffer, and 1.5U AmpliTaq (Applied Biosystems, Foster City, CA), to which 2 μL of the first-round reaction were added. Thermocycling conditions were 95°C for 2 minutes; 30 cycles of 95°C for 30 seconds, 55°C for 30 seconds, and 72°C for 1 minute, followed by 72°C for 10 minutes.

PCR products of the correct size were confirmed by gel electrophoresis, purified using Exosap (USB, Cleveland, OH), and sequenced by dideoxynucleoside-based analysis using a Big Dye terminator kit (Applied Biosystems) and ABI Prism 3100 equipment. Three sequencing reactions were performed on each PCR product using the following primers: RT4 (as above), pol612-632_F (5′-AATTGGGCCTGAAAATCCATA-3′), and pol529-548_F (5′-AAACAATGGCCATTGACAGA-3′). The resulting nucleotide *pol* sequences were analyzed using Sequencher, Version 4.5 (Gene Codes Co, Ann Arbor, MI). A minimum of 4 sequences (2 forward and 2 reverse) from each sample were aligned. To differentiate mixed peaks from background noise, a line was drawn such that 95% of the secondary peaks were below the line. A site is then defined as a "mixed peak" if the secondary peak is above the background line in

at least 3 of 4 sequences. A consensus sequence was made from these sequences and submitted to the Stanford University HIV Drug Resistance Database (http://hivdb.stanford.edu/) for interpretation of drug resistance profiles. In replicate control reactions with this method, we reliably detected mutant sequences when present at or above 20% of total sequence (data not shown).

Viral subtypes were determined from the *pol* sequences with PAUP* version 4.0b10[24] by creating a neighbor-joining phylogenetic tree with reference sequences from the Los Alamos National Laboratory HIV Database (http://www.hiv.lanl.gov/).

Allele-Specific PCR to Detect K103N and Y181C Mutations

A first-round RT-PCR performed with a common primer set was followed by parallel quantitative allele-specific and total HIV copy real-time PCRs. Two different common primer sets were used in duplicate RT-PCRs to minimize amplification failure due to sequence diversity in the primer binding sites. Each RT-PCR was performed in 25 μL with final reagent concentrations of 4 ng/μL each of primers RT1 and RT4 or RT18 and RT21 and 1x Reaction Mix (Invitrogen) to which 0.5 μL SuperScript III RT/Platinum *Taq* mix (Invitrogen) and 5 μL of either extracted viral RNA, no-template controls, or RNA standards (either K103N, Y181C, or wild type) were added. Thermocycling conditions were 45°C for 60 minutes; 95° C for 2 minutes; 10 cycles of 95°C for 30 seconds, 55°C for 30 seconds, and 72°C for 1 minute, followed by 72°C for 10 minutes.

First-round product from the 10-cycle RT-PCR was diluted 1:10, and 5 μL were added to parallel reactions of second-round allele-specific or total HIV copy real-time PCR. Each reaction was performed in 25 μL with final concentrations of 1x SYBR GreenER qPCR SuperMix for ABI Prism (Invitrogen), and 4 ng/μL each of forward and reverse primers. To quantify total HIV copies, we used forward primer: pol612-632_F (5′-AATTGGGCCTGAAAATCCATA-3′) and reverse primer: pol775-800_R (5′-CCCACATCCAGTACTGTTACTGATTT-3′). For allele-specific amplification, the forward primer was the same as for total HIV copy PCR, but the reverse primer was replaced by K103N-rev-mut (5′-CCACATCCAGTACTGTTACTGATTTG-3′) for amplification of K103N mutants and replaced by Y181C-Q23-2-sm (5′-ACATACAAGTCATCCATGTATTGAC-3′) for amplification of Y181C mutants. Parallel reactions of total HIV copy, K103N allele-specific, and Y181C allele-specific real-time PCR were performed on the same first-round PCR product using SYBR green detection on an ABI prism 7900HT.

The quantities of total and drug-resistant HIV copies was estimated by comparing the cycle threshold values of samples of interest with those from 10^4 to 10^{-1} copies per microliter serial dilutions of either wild type, K103N, or Y181C standard RNA template. The proportion of drug-resistant virus was determined by dividing the quantity of drug-resistant sequences, as defined by allele-specific real-time PCR, by the quantity of total HIV sequences, as defined by the total HIV copy real-time PCR. Samples were run in duplicate or quadruplicate. To ensure adequate sequence input to detect at least 2% mutant, assay results for each test in which <20 copies were measured by the total HIV real-time assay were excluded from the analysis. In addition, the total HIV copies in each replicate assay were added together to calculate the total viral copies tested for each sample. Samples in which the sum total HIV tested was less than 50 copies were excluded. For each independent allele-specific assay run on a sample, if the measured drug-resistant copy number was between 0 and 1, the result of that replicate was set equal to zero. The proportion of drug-resistant virus was calculated for each reaction, and if it was below the lower limit of the assay (0.2% for K103N and 2% for Y181C, Fig. 1), the proportion for that reaction was set to zero. The average proportion of drug resistant to wild type was calculated by averaging the replicate assay results from each sample tested.

Statistical Analysis

All analyses were intent-to-treat and were performed using Stata version 9.2 (Stata Corp, College Station, TX). Median $\log_{10}$ plasma viral loads and CD4 counts were compared between treatment arms using the Mann–Whitney *U* test. Fisher exact tests and univariate logisitic regression were used to determine correlates of NVP resistance.

RESULTS

Study Population

Pregnant women were randomized at 34 weeks gestation to receive either combination ZDV/sdNVP or short-course HAART for prevention of MTCT. Women were included in this substudy if they had a plasma sample available from 3 months after treatment cessation. Of the 28 women originally randomized to ZDV/sdNVP, 4 were lost to follow-up.[19] Of the remaining 24 women, 23 women had plasma samples collected 3 months after treatment cessation (3 months postpartum). Of the 30 women originally randomized to the HAART arm, 6 were lost to follow-up and 2 had adverse events.[19] Of the remaining 22 women, 17 had plasma samples available from 3 months after treatment cessation (9 months postpartum).

At enrollment, the characteristics of the women studied here were comparable between arms. Median CD4 count at 32 weeks gestation was 354 [interquartile range (IQR) 295–430] in the ZDV/sdNVP arm and 304 (IQR 261–404) in the HAART arm ($P = 0.18$). Median $\log_{10}$ HIV-1 RNA plasma viral load at 32 weeks gestation was 4.76 (IQR 4.24–5.25) and 4.87 (IQR 4.6–5.03) copies per milliliter in the ZDV/sdNVP and HAART arms, respectively ($P = 0.67$).

Resistance Undetectable by Population-Based Sequencing

We used population-based sequencing to examine resistance to any of the nucleoside reverse transcriptase inhibitors (NRTIs) and the NNRTIs at 3 months after ZDV/sdNVP or HAART cessation. To ensure that we tested an adequate number of viral copies to detect resistance at the >20% level possible with population-based sequencing, we estimated the number of HIV copies we added to each RT-PCR for amplification before sequencing. This estimate was based on the plasma viral load measured by the Gen-Probe HIV-1 viral load assay (Table 1). A median of 528 viral RNA copies (range 5–2884) was put into the first-round RT-PCR reaction. Of the 40 samples tested, we were not able to amplify the viral RNA from 1 woman in the HAART arm. However, the estimated RNA input from that sample was ≤5 copies. Of the remaining 39 samples tested, 3 (7.7%) had an input of fewer than 50 viral RNA copies, 7 (18%) had an input of 50–100 copies, whereas 29 (74%) had an input of over 100 copies based on original plasma viral load data. Although the true copy input may have been 2- to 5-fold lower due to loss of RNA during extraction (data not shown), even with a 5-fold loss, we estimate to have sequenced at least 10 copies in 92% of samples tested.

The population-based sequencing method used here provides sequence data from codon 30 to codon 225 of *RT* and is therefore capable of detecting the majority of mutations known to confer resistance to any of the NRTIs or NNRTIs. By this method, none of the samples in either of the 2 treatment arms showed detectable levels of resistance to any of the NRTIs or NNRTIs.

Phylogenetic analysis of this *pol* sequence data indicates that the majority (72%) of women were infected with subtype Aviruses, whereas fewer, 18%, 5%, 2.6%, and 2.6% were infected with subtypes D, C, G, and A/C recombinant, respectively (Table 1).

Frequency of K103N and Y181C Resistance Mutations Detected by Allele-Specific PCR

We used allele-specific PCR to examine low levels of K103N and Y181C viral variants in the samples from 3 months after treatment cessation. We limited this analysis to the 27 samples

(16 and 11 in the ZDV/sdNVP and HAART arms, respectively) in which at least 50 cumulative viral RNA copies were tested in the 2–4 independent RT-PCRs (see Methods and Table 1).

The levels of K103N detected by allele-specific PCR ranged from 0.8% to 3% of total virus, and levels of Y181C ranged from 1.1% to 14.2% (Table 1). The levels reported are averages of the repeated allele-specific PCR measurements on each sample. For each replicate, we set the proportion of resistance to zero if it was below the lower limit of the assay as defined by replicate testing of wild-type controls: 0.2% and 2% for the K103N and Y181C assays, respectively (Fig. 1). Therefore, in 2 cases in which a sample had replicate results both above and below the lower limit for a single allele-specific assay, the average reported is less than the 2% lower limit for Y181C (Table 1). For example, the results for duplicate Y181C assays on virus from patient 569 were below detection (set to zero) for duplicate 1 and 2.2% for duplicate 2. Therefore, we report the estimated proportion of Y181C virus in that patient to be 1.1%: the average of the duplicate tests.

K103N mutations were detected by allele-specific PCR in 5 of the 27 women (19%) included here (Table 2). All 5 women with K103N resistance mutations were in the ZDV/sdNVP arm, whereas there were no women with detectable K103N viral variants in the HAART arm. Y181C mutations were detected by allele-specific PCR in 11 of the 27 women (41%) (Table 2). Nine of 16 women (56%) in the ZDV/sdNVP arm and 2 of 11 women (18%) in the HAART arm had viral variants with detectable levels of Y181C. Only 2 of the women had detectable levels of both K103N and Y181C mutations.

ZDV/sdNVP Compared With HAART Results in a Significantly Higher Frequency of NNRTI Resistance

The frequency of detectable K103N and/or Y181C viral variants 3 months after treatment cessation in the ZDV/sdNVP arm was 75%, whereas only 18% in the HAART arm (Table 2). The difference in frequency of drug resistance in the 2 arms was statistically significant (P = 0.006, Fisher exact test). The odds of resistance was 13.5 times higher in the ZDV/sdNVP arm compared with the HAART arm, according to a univariate logistic regression model (P = 0.007, Table 3). To rule out confounding, we also performed univariate logistic regression with the following potential correlates: $\log_{10}$ plasma viral load at 32 weeks gestation, CD4 count at 32 weeks gestation, $\log_{10}$ plasma viral load at 3 months posttreatment, and viral subtype (Table 3), none of which were statistically significant predictors of resistance in these women. In addition, median $\log_{10}$ plasma viral and CD4 counts were similar at baseline in women with and without resistance at 3 months posttreatment according to a Mann–Whitney U test (data not shown).

Resistance Can Persist in Women for 1-Year Posttreatment

We used allele-specific PCR to examine whether the low levels of K103N and Y181C viral variants detected at 3 months after treatment cessation were also present at 6 or 12 months posttreatment. We limited this analysis to the 14 women (12 and 2 in the ZDV/sdNVP and HAART arms, respectively) in whom we detected resistance at 3 months after treatment cessation (Table 2). In the 5 women with detectable K103N mutations at 3 months posttreatment, K103N levels fell below detection in 3 of the women, whereas the other 2 women (40%) still had detectable K103N mutations at 12 months posttreatment (Table 4). In 1 woman (ID# 654), levels were variable over time, but K103N was consistently detected in both #654 and in #629 at 3, 6, and 12 months post-ZDV/sdNVP.

Eight of the 9 women (89%) in the ZDV/sdNVP arm with low levels of Y181C at 3 months posttreatment had detectable levels of Y181C at 12 months posttreatment. In most cases, the levels of Y181C were quite stable at all 3 time points, varying at most by ~3-fold over time

but showing no evidence of consistent decline. In women treated with HAART, 1 of the 2 women with previously detectable Y181C mutations still had detectable levels of Y181C variants when follow-up ended, 6 months after treatment cessation (Table 5).

DISCUSSION

In this randomized trial, the use of short-course HAART led to a significantly lower prevalence of NNRTI resistance compared with the ZDV/sdNVP regimen ($P = 0.007$). To our knowledge, this is the first time the prevalence of resistance after these 2 regimens has been compared in a randomized trial. As ZDV/sdNVP is recommended in nonimmunosuppressed HIV-1–infected women to prevent MTCT per World Health Organization guidelines, and short-course HAART is under consideration,[3,19] this study provides critical information when assessing the relative merits of these antiretroviral interventions.

In this study, detectable levels of resistance ranged from 0.8% to 14.2% of an individual's virus population and were detectable by a sensitive allele-specific PCR assay but were not detectable by population-based sequencing at 3 months posttreatment. The discordance between the results from the different assays used reflects the difference in their sensitivity: the population-based sequencing assay only reliably detects mutations that comprise >20% of the virus population, whereas the K103N and Y181C allele-specific PCR assays detect resistance down to 0.2% and 2%, respectively.

Here we show no resistance detectable by sequencing despite the fact that we estimate to have sequenced a minimum of 10–50 copies in 92% of samples tested. In addition, independent PCR and sequencing reactions were repeated up to 3 times on 82% of these samples, and the results were identical (data not shown). The fact that none of the women in our study had levels of resistance detectable with a sequencing assay is somewhat surprising. Previous studies of both sdNVP alone and ZDV/sdNVP have shown that NNRTI resistance is detectable by sequencing in 15%–69% of women at 4–8 weeks postpartum.[11] The lack of resistance detectable by sequencing in our study could result from the fact that the majority of women in our cohort were infected with subtype A (72%) or subtype D (18%) virus, and previous studies suggest that lower rates of NVP resistance occur in subtypes A and D compared with C.[25] There is also some evidence that resistance is more common in women with low baseline CD4 counts and high baseline viral load, and our cohort excluded women with CD4 counts <200 cells per cubic millimeter.[12,26] In addition, we sampled at a time point later than previous studies, and viral variants with resistance mutations have been shown to wane over time.[26–30]

Seventy-five percent of the women in the ZDV/sdNVP arm had viral variants with K103N or Y181C mutations detectable by our allele-specific PCR assay, whereas only 18% of women in the HAART arm had detectable levels of resistant virus ($P = 0.007$, Tables 2 and 3). The fact that the study design was a randomized trial minimizes the chances that the prevalence of baseline resistance differed in the 2 arms. The reduction in risk of resistance after HAART cessation compared with ZDV/sdNVP may derive from the fact that suppression of plasma HIV-1 RNA is consistently several $\log_{10}$ greater with HAART compared with ZDV/sdNVP.[19] As a result, the levels of replicating virus during the period when drug levels wane immediately after treatment cessation is lower with HAART compared with ZDV/sdNVP. In addition, the use of 3TC in the HAART regimen, which has a longer half-life than ZDV, decreases the amount of time that NVP may be found as the only active drug in plasma after treatment cessation. The effect of a second drug during NVP cessation is supported by other studies that have shown that the addition of a "tail" of antiretrovirals after cessation of NVP-based treatments reduces the risk of resistance (reviewed in[31]). Studies that included 3–7 days of ZDV plus 3TC after the use of sdNVP showed reduced rates of resistance compared with sdNVP alone.[32,33] Therefore, it is unknown whether the addition of a "tail" concurrent with

treatment cessation of ZDV/sdNVP would make the prevalence of resistance in the 2 regimens studied here more similar. Our data suggest that even without a "tail" added during HAART cessation, the presence of 3TC alone may substantially reduce NVP resistance. This may be important in cases when a tail is not applied, such as unexpected treatment cessation.

Previous studies suggest that the emergence of resistance to NVP after treatment to prevent MTCT may have consequences for women who later require long-term HAART.[12,13] Therefore, it is important to determine whether resistance persists over time. Although data from studies of resistance using standard genotyping assays suggest that resistance to NVP wanes over time,[26,34] studies that utilize more sensitive assays suggest that resistance can persist at low levels for more than 1 year postpartum.[27,28,35] Our data confirm this suggesting that in women with detectable levels of resistance at 3 months postpartum, resistance can persist at 6 and 12 months posttreatment (Tables 4 and 5). Of note, variants with Y181C mutations, which are more common in this cohort than K103N mutations, persisted above detection at 12 months posttreatment in over 80% of the women, with little evidence that they declined during this period. Thus, Y181C may be of particular concern in terms of its effect on future treatment options.

The data presented here provides strong evidence that short-course HAART results in lower rates of antiretroviral resistance compared with the standard ZDV/sdNVP regimen. However, the relative effects of these regimens on transmission rates remain less clear. Preliminary data from ongoing studies in Kenya and Tanzania suggest that 6 months of HAART during breastfeeding reduces transmission rates from the expected 25%–48% without treatment to 5% by 6 months postpartum.[3,5,6] A short course of ZDV/3TC plus sdNVP have a documented transmission rate of 6.6% at 6 months postpartum.[36] Given the imprecision of comparing data from distinct cohorts, 2 randomized clinical trials are currently underway to more accurately determine whether taking HAART during breastfeeding reduces transmission compared with standard short-course regimens.[3,7]

When considering antiretroviral strategies to reduce breastfeeding transmission, data on transmission rates must be balanced with factors such as resistance, safety, feasibility, and adherence. These issues, and the optimal approaches to PMTCT, are complex and are discussed in more detail elsewhere.[1,3,19] A previous study of our cohort showed that 5 of 26 women (19%) randomized to HAART prematurely discontinued treatment either due to adverse events or inconvenience,[19] suggesting adherence can be low with HAART. The current study was limited to 17 women on HAART, and we acknowledge that this analysis could be biased toward a group of women with good adherence. The reduced risk of resistance with HAART (even without an added tail) suggests that the use of HAART, although breastfeeding may have fewer implications for later treatment options compared with the combination ZDV/sdNVP regimen, as NNRTI resistance after treatment prophylaxis has been implicated to reduce the effectiveness of later treatment with NNRTI-based HAART.[12,13] Therefore, the data presented here should be considered as a benefit of HAART when balancing the safety, efficacy, and feasibility of different strategies currently being tested to reduce breastfeeding transmission. These data are encouraging for settings in which adding a tail of 3TC and ZDV during HAART cessation is not feasible. However, the fact that the use of HAART did not completely eliminate resistance to NVP emphasizes that, when possible, the addition of ZDV and 3TC during treatment cessation may be beneficial.

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References

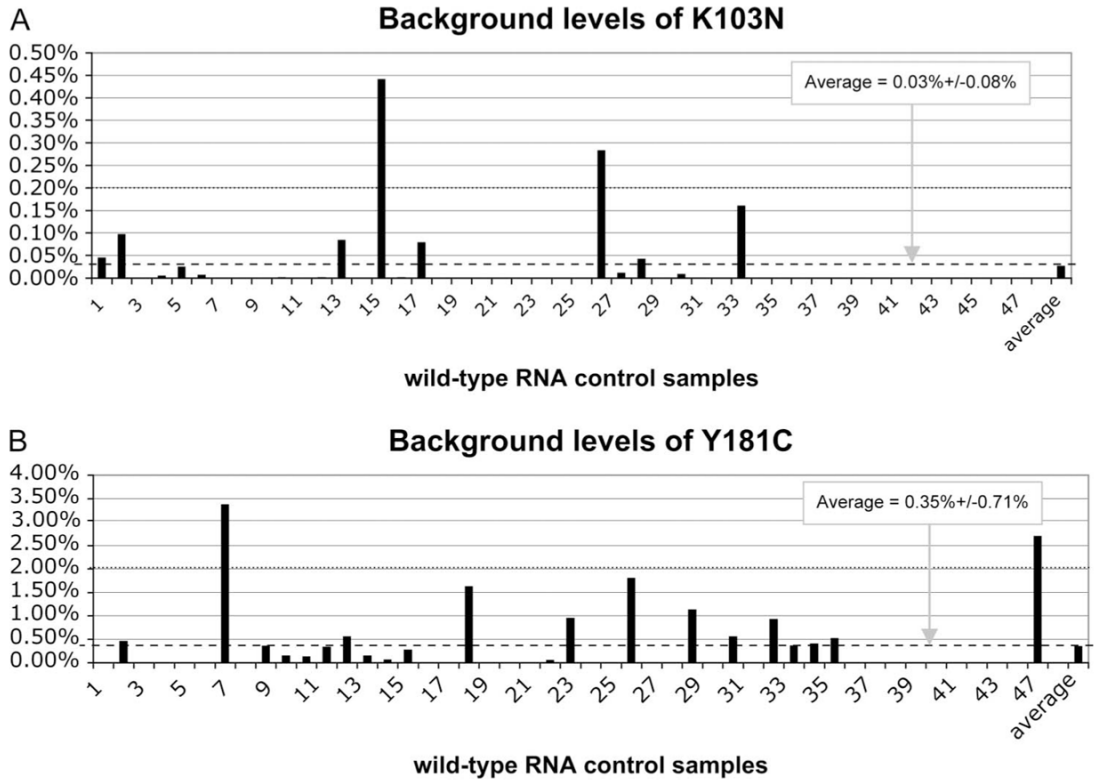
1. World Health Organization. Antiretroviral drugs for treating pregnant women and preventing HIV infection in infant: towards universal access. [Accessed July 17, 2006]. Available at: http://www.who.int/hiv/pub/guidelines/pmtctguidelines3.pdf
2. Gray GE, Saloojee H. Breast-feeding, antiretroviral prophylaxis, and HIV. N Engl J Med 2008;359:189–191. [PubMed: 18525037]
3. Mofenson LM. Antiretroviral prophylaxis to reduce breast milk transmission of HIV type 1: new data but still questions. J Acquir Immune Defic Syndr 2008;48:237–240. [PubMed: 18545160]
4. Kumwenda NI, Hoover DR, Mofenson LM, et al. Extended antiretroviral prophylaxis to reduce breast-milk HIV-1 transmission. N Engl J Med February 4 2008;359:119–129.
5. Thomas, T.; Masaba, R.; Ndivo, R., et al. Prevention of mother-to-child transmission of HIV-1 among breastfeeding mothers using HAART: the Kisumu Breastfeeding Study, Kisumu, Kenya, 2003–2007. Paper presented at: 15th Conference on Retroviruses and Opportunistic Infections; February 4, 2008; Boston, MA.
6. Kilewo, C.; Karlsson, K.; Ngarina, M., et al. Prevention of mother-to-child transmission of HIV-1 through breastfeeding mothers prophylactically with triple antiretroviral therapy in Dar es Salaam, Tanzania—The MITRA plus study. Presented at: 4th International AIDS Society Conference on HIV Pathogenesis and Treatment; July 22–25, 2007; Sydney, Australia.
7. Kourtis AP, Jamieson DJ, de Vincenzi I, et al. Prevention of human immunodeficiency virus-1 transmission to the infant through breastfeeding: new developments. Am J Obstet Gynecol 2007;197:S113–S122. [PubMed: 17825642]
8. Oyugi JH, Byakika-Tusiime J, Ragland K, et al. Treatment interruptions predict resistance in HIV-positive individuals purchasing fixed-dose combination antiretroviral therapy in Kampala, Uganda. AIDS 2007;21:965–971. [PubMed: 17457090]
9. Hare CB, Mellors J, Krambrink A, et al. Detection of nonnucleoside reverse-transcriptase inhibitor-resistant HIV-1 after discontinuation of virologically suppressive antiretroviral therapy. Clin Infect Dis 2008;47:421–424. [PubMed: 18558886]
10. Lehman DA, Farquhar C. Biological mechanisms of vertical human immunodeficiency virus (HIV-1) transmission. Rev Med Virol 2007;17:381–403. [PubMed: 17542053]
11. Arrive E, Newell ML, Ekouevi DK, et al. Prevalence of resistance to nevirapine in mothers and children after single-dose exposure to prevent vertical transmission of HIV-1: a meta-analysis. Int J Epidemiol 2007;36:1009–1021. [PubMed: 17533166]
12. Jourdain G, Ngo-Giang-Huong N, Le Coeur S, et al. Intrapartum exposure to nevirapine and subsequent maternal responses to nevirapine-based antiretroviral therapy. N Engl J Med 2004;351:229–240. [PubMed: 15247339]
13. Lockman S, Shapiro RL, Smeaton LM, et al. Response to antiretroviral therapy after a single, peripartum dose of nevirapine. N Engl J Med 2007;356:135–147. [PubMed: 17215531]
14. Johnson JA, Li JF, Wei X, et al. Minority HIV-1 drug resistance mutations are present in antiretroviral treatment-naive populations and associate with reduced treatment efficacy. PLoS Med 2008;5:e158. [PubMed: 18666824]
15. Richman D, Shih CK, Lowy I, et al. Human immunodeficiency virus type 1 mutants resistant to nonnucleoside inhibitors of reverse transcriptase arise in tissue culture. Proc Natl Acad Sci U S A 1991;88:11241–11245. [PubMed: 1722324]
16. Richman DD, Havlir D, Corbeil J, et al. Nevirapine resistance mutations of human immunodeficiency virus type 1 selected during therapy. J Virol 1994;68:1660–1666. [PubMed: 7509000]
17. Cressey TR, Jourdain G, Lallemant MJ, et al. Persistence of nevirapine exposure during the postpartum period after intrapartum single-dose nevirapine in addition to zidovudine prophylaxis for

the prevention of mother-to-child transmission of HIV-1. J Acquir Immune Defic Syndr 2005;38:283–288. [PubMed: 15735445]

18. Perez H, Vignoles M, Laufer N, et al. Low rate of emergence of nevirapine and lamivudine resistance after post-partum interruption of a triple-drug regimen. Antivir Ther 2008;13:135–139. [PubMed: 18389908]
19. Chung MH, Kiarie JN, Richardson BA, et al. Highly active antiretroviral therapy versus zidovudine/nevirapine effects on early breast milk HIV type-1 RNA: a phase II randomized clinical trial. Antivir Ther 2008;13:799–807. [PubMed: 18839781]
20. Lehman DA, Chung MH, John-Stewart GC, et al. HIV-1 persists in breast milk cells despite antiretroviral treatment to prevent mother-to-child transmission. AIDS 2008;22:1475–1485. [PubMed: 18614871]
21. DeVange Panteleeff D, Emery S, Richardson BA, et al. Validation of performance of the Gen-probe human immunodeficiency virus type 1 viral load assay with genital swabs and breast milk samples. J Clin Microbiol 2002;40:3929–3937. [PubMed: 12409354]
22. Emery S, Bodrug S, Richardson BA, et al. Evaluation of performance of the Gen-probe human immunodeficiency virus type 1 viral load assay using primary subtype A, C, and D isolates from Kenya. J Clin Microbiol 2000;38:2688–2695. [PubMed: 10878065]
23. ANRS AC11 Resistance Study Group. PCR and sequencing procedures: HIV-1, version. Feb2008 [Accessed May 15, 2008]. Available at: http://www.hivfrenchresistance.org/ANRS-procedures.pdf
24. PAUP: phylogenetic analysis using parsimony [computer program]. Champaign, IL: Natural History Survey; 1991.
25. Eshleman SH, Hoover DR, Chen S, et al. Nevirapine (NVP) resistance in women with HIV-1 subtype C, compared with subtypes A and D, after the administration of single-dose NVP. J Infect Dis 2005;192:30–36. [PubMed: 15942891]
26. Eshleman SH, Mracna M, Guay LA, et al. Selection and fading of resistance mutations in women and infants receiving nevirapine to prevent HIV-1 vertical transmission (HIVNET 012). AIDS 2001;15:1951–1957. [PubMed: 11600822]
27. Loubser S, Balfe P, Sherman G, et al. Decay of K103N mutants in cellular DNA and plasma RNA after single-dose nevirapine to reduce mother-to-child HIV transmission. AIDS 2006;20:995–1002. [PubMed: 16603851]
28. Palmer S, Boltz V, Martinson N, et al. Persistence of nevirapine-resistant HIV-1 in women after single-dose nevirapine therapy for prevention of maternal-to-fetal HIV-1 transmission. Proc Natl Acad Sci U S A 2006;103:7094–7099. [PubMed: 16641095]
29. Flys TS, Donnell D, Mwatha A, et al. Persistence of K103N-containing HIV-1 variants after single-dose nevirapine for prevention of HIV-1 mother-to-child transmission. J Infect Dis 2007;195:711–715. [PubMed: 17262714]
30. Kassaye S, Lee E, Kantor R, et al. Drug resistance in plasma and breast milk after single-dose nevirapine in subtype C HIV type 1: population and clonal sequence analysis. AIDS Res Hum Retroviruses 2007;23:1055–1061. [PubMed: 17725424]
31. Dao H, Mofenson LM, Ekpini R, et al. International recommendations on antiretroviral drugs for treatment of HIV-infected women and prevention of mother-to-child HIV transmission in resource-limited settings: 2006 update. Am J Obstet Gynecol 2007;197:S42–S55. [PubMed: 17825650]
32. Chaix ML, Ekouevi DK, Rouet F, et al. Low risk of nevirapine resistance mutations in the prevention of mother-to-child transmission of HIV-1: Agence Nationale de Recherches sur le SIDA Ditrame Plus, Abidjan, Cote d'Ivoire. J Infect Dis 2006;193:482–487. [PubMed: 16425126]
33. McIntyre, J.; Martinson, N.; Gray, G., et al. Addition of short course combivir (CBV) to single dose viramune (sdNVP) for the prevention of mother to child transmission (pMTCT) of HIV-1 can significantly decrease the subsequent development of maternal and paediatric NNRTI-resistant virus. Paper presented at: The 3rd IAS Conference on HIV Pathogenesis and Treatment; July 24–27, 2005; Rio de Janeiro, Brazil.
34. Eshleman SH, Guay LA, Wang J, et al. Distinct patterns of emergence and fading of K103N and Y181C in women with subtype A vs. D after single-dose nevirapine: HIVNET 012. J Acquir Immune Defic Syndr 2005;40:24–29. [PubMed: 16123677]

35. Flys T, Nissley DV, Claasen CW, et al. Sensitive drug-resistance assays reveal long-term persistence of HIV-1 variants with the K103N nevirapine (NVP) resistance mutation in some women and infants after the administration of single-dose NVP: HIVNET 012. J Infect Dis 2005;192:24–29. [PubMed: 15942890]

36. Tonwe-Gold B, Ekouevi DK, Viho I, et al. Antiretroviral treatment and prevention of peripartum and postnatal HIV transmission in West Africa: evaluation of a two-tiered approach. PLoS Med 2007;4:e257. [PubMed: 17713983]



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Fales positive rates				
	lower limit of detection			
assay	≥2%	≥1%	≥0.2%	≥0.1%
K103N	0%	0%	**4%**	8%
Y181C	**4%**	10%	33%	40%

FIGURE 1.

Background levels of K103N and Y181C allele-specific PCR assays. Results of allele-specific PCR on 48 replicates of wild-type RNA template. Total HIV copy number was quantified by real-time PCR, and an average of 379 copies (range: 76–779) of wild-type RNA was tested in each reaction. A, Results of K103N allele-specific PCR on wild-type template. The average background across all 45 samples is shown with a dashed line. The lower limit of 0.2%, defined by a <5% false-positive rate is shown with a dotted line. B, Results of Y181C allele-specific PCR on wild-type template. The average background across all 45 samples is shown with a dashed line. The lower limit of 2%, defined by a <5% false-positive rate is shown with a dotted line. C, The false-positive rates at different lower limits of detection are shown. (figure modeled after Palmer et al. *AIDS*. 2006;20:701–710).

TABLE 1

Cohort Characteristics and Percentage of K103N and Y181C Detected by Allele-Specific PCR

	32 Weeks Gestation		ARV Regimen	3 Months Post ARV			Drug Resistance ~3 Months Post ARV		
ID No.	Log PlasmaViral Load (Copies/mL)	CD4Count	Treatment Arm	Days Since Last ARV	Log Plasma Viral Load (Copies/ mL)	Viral Subtype*	Copies of Virus Tested†	% K103N‡	% Y181C‡
518	5.98	283	ZDV/sdNVP	91	5.55	A	126	bd	bd
520	4.18	264	ZDV/sdNVP	91	3.87	D	164	bd	bd
523	5.25	477	ZDV/sdNVP	93	5.61	A	2526	bd	bd
541	4.76	354	ZDV/sdNVP	91	4.93	D	219	bd	**12**
543	4.87	333	ZDV/sdNVP	91	3.92	A	371	bd	**3**
544	4.28	321	ZDV/sdNVP	93	4.60	A	175	**3**	bd
548	4.81	309	ZDV/sdNVP	90	5.04	A	56	bd	bd
569	4.98	295	ZDV/sdNVP	93	5.10	A	211	bd	**1.1**§
579	4.34	461	ZDV/sdNVP	93	4.94	A	388	bd	**10.3**
583	5.85	212	ZDV/sdNVP	97	5.60	A	2021	bd	**11.7**
601	4.24	360	ZDV/sdNVP	94	4.03	D	200	**0.9**	**7.7**
604	4.37	458	ZDV/sdNVP	91	4.25	A	131	bd	**14.2**
609	5.37	295	ZDV/sdNVP	96	5.66	A	768	bd	**4.9**
629	5.16	365	ZDV/sdNVP	NA	5.34	A	2013	**1.2**	**1.4**§
654	5.79	407	ZDV/sdNVP	94	3.86	A	66	**3**	bd
656	4.00	431	ZDV/sdNVP	124	4.42	A	131	**0.8**	bd
555	5.85	312	HAART	89	6.08	D	971	bd	bd
561	4.88	404	HAART	115	4.86	A	688	bd	bd
573	3.47	273	HAART	96	5.05	A	602	bd	**4.1**
585	4.87	316	HAART	115	5.28	D	90	bd	bd
590	4.32	304	HAART	91	4.78	A	419	bd	bd
591	4.91	256	HAART	78	4.74	D	79	bd	bd
616	5.06	320	HAART	214	5.53	C	204	bd	bd
627	5.44	261	HAART	49	5.38	C	102	bd	bd
630	4.81	275	HAART	84	4.02	A/C	381	bd	bd
632	4.89	229	HAART	95	3.80	A	146	bd	**2**
642	4.43	267	HAART	91	4.82	A	103	bd	bd

ARV, antiretroviral; bd, below detection of the allele-specific PCR assay; NA, not available.

*Viral subtype based on neighbor-joining phylogenetic tree.

†Copies of virus tested based on average total HIV copy real-time PCR.

‡Percentage of mutant reported is an average of replicate tests. Bold numbers indicate resistance was detected.

§%Y181C average is below the level of detection due to replicate testing results falling above and below the limit of detection.

TABLE 2

Frequency of K103N and Y181C Mutations in ZDV/sdNVP and HAART Arms

ARV Regimen	Women, n	K103N, n (%)	Y181C, n (%)	K103N or Y181C, n (%)
ZDV/sdNVP	16	5 (31)	9 (56)	12 (75)
HAART	11	0 (0)	2 (18)	2 (18)
Total	27	5 (19)	11 (41)	14 (52)

ARV, antiretroviral.

TABLE 3

Univariate Logistic Regression With Potential Correlates of Resistance

Potential Correlate	OR (95% CI)	*P*
Treatment ZDV/sdNVP vs HAART	13.5 (2.0 to 90.7)	0.007
Log_{10} plasma viral load 32 weeks gestation	0.5 (0.13 to 1.88)	0.304
CD4 count at 32 weeks gestation*	1.3 (0.75 to 2.17)	0.376
Log plasma viral load at 3 months post ARV	0.39 (0.11 to 1.42)	0.152
Subtype†	0.22 (0.042 to 1.15)	0.073

ARV, antiretroviral; CI, confidence interval; OR, odds ratio.

*CD4 count categorized into intervals of 50 cells per cubic millimeter.

†Subtype A was the reference group.

TABLE 4

The Frequency of K103N Mutations at 3, 6, and 12 Months Posttreatment

ID No.	Treatment Arm	% K103N*		
		3m†	6m	12m
544	ZDV/sdNVP	3	0.3	bd
601	ZDV/sdNVP	0.9	—	bd
629	ZDV/sdNVP	1.2	0.4	0.8
654	ZDV/sdNVP	3	14.0	0.2
656	ZDV/sdNVP	0.8	bd	bd

bd, below detection of the allele-specific PCR assay.

*% K103N reported is an average of replicate tests.

†Three-month data are from Table 1.

TABLE 5

The Frequency of Y181C Mutations at 3, 6, and 12 Months Posttreatment

ID No.	Treatment Arm	%Y181C*		
		3m†	6m	12m
541	ZDV/sdNVP	12	8.5	8.5
543	ZDV/sdNVP	3	5.6	2.4
569	ZDV/sdNVP	1.1	2.2	2.9
579	ZDV/sdNVP	10.3	12.1	11.2
583	ZDV/sdNVP	11.7	11.4	11.2
601	ZDV/sdNVP	7.7	—	2.4
604	ZDV/sdNVP	14.2	—	7.7
609	ZDV/sdNVP	4.9	—	9.8
629	ZDV/sdNVP	1.4	bd	bd
573	HAART	4.1	4.9	—
632	HAART	2	bd	—

bd, below detection of the allele-specific PCR assay.

*% Y181C reported is an average of replicate tests.

†Three-month data are from Table 1.