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Selection of HIV Resistance associated with Antiretroviral Therapy (ART) Initiated due to Pregnancy and Suspended Postpartum

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Abstract

Objective—Compare the risk of HIV-drug-resistance in women stopping suppressive nelfinavir (NFV) or nevirapine (NVP)-based-ART after pregnancy.

Methods—Specimens collected after stopping ART were tested for drug-resistance by an oligonucleotide ligation assay (OLA) and consensus sequencing. When postpartum drug-resistance was detected, specimens obtained at study entry and during ART were evaluated.

Results—16/38 women with ART-induced suppression of viral replication suspended ART postpartum. Resistance mutations were detected in 75% who stopped NFV- and 50% who stopped NVP-ART. M184V, associated with 3TC-resistance, was more frequent among those randomized to NFV- compared to NVP-ART (6/8 vs. 1/8; P=0.04), and NNRTI-resistance was detected in 4/8 stopping NVP-ART.

Conclusions—HIV-drug-resistance was frequently observed among women who stopped suppressive NVP- or NFV-ART postpartum. This suggests that NFV-ART may have suboptimal potency; that staggering discontinuation of NVP-ART may be warranted; and/or ART-adherence may be lax in women who choose to stop ART postpartum.

Keywords

HIV-1; Antiretroviral drug resistance; Antiretroviral discontinuation; Pregnancy; Oligonucleotide ligation assay (OLA); Minority genotypes; Nelfinavir; Nevirapine; Lamivudine; Zidovudine

Introduction

ART is extremely effective in the prevention of mother-to-child-transmission of HIV (pMTCT),¹ and is frequently given during pregnancy to women who otherwise would not qualify for treatment.² Stopping ART after immune reconstitution has been linked with increased morbidity and mortality.^{3,4} Nevertheless, pregnant women who do not qualify for

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ART based on treatment guidelines² often choose to discontinue ART after the birth of their infant.⁵

A few studies have evaluated the selection of drug-resistance mutations when women stop ART postpartum.^{5,6} These studies suggest that cessation of ART postpartum may select drug-resistant mutants. To further investigate the risk of stopping ART postpartum, we assessed and compared the frequency of HIV drug-resistance in women randomized between nelfinavir (NFV)- and nevirapine (NVP)-based regimens for pMTCT and who discontinued ART postpartum.

Materials and Methods

Study design and participant specimens

Pregnant women with plasma HIV RNA loads >1000 copies/mL were enrolled into Pediatric AIDS Clinical Trials Group protocol 1022 (P1022).⁷ The study aimed to determine whether a protease inhibitor (PI)- or non-nucleoside reverse transcriptase inhibitor (NNRTI)-based regimen would be more effective or better tolerated in women initiating therapy during pregnancy. HIV-infected antiretroviral (ARV)-naïve pregnant women who consented to enrollment were randomized to NFV or NVP in combination with zidovudine (ZDV) and lamivudine (3TC) for pMTCT. Women randomized to NVP (supplied as Viramune™ by Boehringer Ingelheim Pharmaceuticals, Inc) were administered 200 mg by mouth once daily for 14 days, then the frequency was increased to 200mg twice daily as tablets or suspension. Those randomized to NFV (supplied as Viracept by Agouron Pharmaceuticals, Inc) were given 1250mg orally twice daily, given as five 250 mg tablets ("older" formulation). In addition, women in both study arms were given ZDV 300 mg and 3TC 150 mg orally twice a day, which could be administered as Combivir™. NFV and NVP were considered to be the safest protease inhibitor (PI) and non-nucleoside reverse transcriptase inhibitors (NNRTI), respectively, for pregnant women at the time. Enrollment into P1022 was discontinued early due to greater than expected toxicity in the NVP group.⁷

Participants eligible for this ART-resistance substudy were ART-naïve at enrollment into P1022, had HIV replication suppressed to <400 copies/mL during study ART, and discontinued ART postpartum. Women continued with study visits until 2 years postpartum, even if study ART was discontinued. Specimens were collected between September 2002 and October 2005, with women discontinuing ART an average of 43 weeks (range 12–101) following delivery. The first available specimen with plasma virus rebound >1000 c/mL, following documented suppression of plasma HIV RNA <400 c/mL, was evaluated for drug-resistance by OLA of both plasma and PBMC. In addition, plasma viral genotype was determined by consensus sequencing. If drug-resistance was detected by either method, then specimens from study entry and intermediate visits were also evaluated for resistance.

Genotypic Testing of Plasma

RNA extracted from the plasma was reverse transcribed and HIV *pol* was PCR amplified and sequenced using the ViroSeq HIV-1 Genotyping System (Abbott Molecular, Des Plaines, IL) per manufacturers instructions. Consensus sequences spanned from protease codon 1 through at least reverse transcriptase codon 225. Our laboratory was certified to perform ViroSeq Genotyping continuously from 2001–2009 by the Viral Quality Assurance (VQA) Program of the National Institutes of Health (NIH). Mutations in HIV-1 Group M subtype were identified using the Stanford HIV Drug Resistance Database.⁸ The nucleic acid sequence was reported to GeneBank, accession # (submission in process).

PCR products from ViroSeq plasma preparations were submitted to a second round of PCR, as described below, prior to OLA.

HIV DNA Quantification and Amplification

DNA was extracted from cryopreserved PBMC using the Puregene Cell and Tissue Kit (Gentra Systems, Minneapolis, MN). DNA concentrations were measured by OD at 260nm and stored at -20°C . Specimens with sufficient DNA had the HIV DNA concentrations assessed by real-time PCR of the *gag* region.⁹

To prepare specimens for OLA testing, PCR was carried out on DNA as previously described¹⁰ with approximately 100 HIV DNA copies per reaction, using PRA and RTA first round primers.¹¹ The first round PCR amplicon from DNA and the ViroSeq amplicon were separately submitted to a second round PCR using primers PRB and RT3.¹¹ The amplicons from the second round PCR, an 1193-bp DNA fragment encoding most of *pol*, were visualized in a 1.2% agarose gel with ethidium bromide staining.

OLA Testing of Plasma and PBMC

An OLA was performed on all amplicons derived from plasma and PBMC using probes that detect mutations associated with high-level drug-resistance to NFV, codons D30N (AAT) and L90M (ATG), nucleoside reverse transcriptase inhibitors (NRTI) at codons K70R (AGA), M184V (GTG), and T215F/Y (TTC, TAC), and non-nucleoside reverse transcriptase inhibitors (NNRTI) at codons K103N (AAC), Y181C (TGT), and G190A (GCA) as described.⁹ The relative concentration of resistant virus was estimated using a standard curve of mutant mixed with wild type virus at the following concentrations: 0%, 2%, 5%, and 100%. Specimens were classified as mutant at the specific codon if the optical density (OD) exceeded the 2% mutant standard for RT codons 103, 181, 190, and 184 and PR codon 90, and the 5% for RT codons 70 and 215, and PR codon 30. Reactions with a mutant OD below the mutant cutoff and a wildtype OD less than half the OD of the wildtype control were considered indeterminate. All participant's specimens and OLA controls were analyzed in duplicate. Control plasmids are described in the OLA manual.¹¹

Pharmacokinetics and Adherence

Intensive blood sampling of women to evaluate the pharmacokinetics of NFV and NVP during pregnancy was conducted in both study arms. Blood sampling occurred at 34 ± 2 weeks gestation and 8 ± 2 weeks postpartum, and was collected predose, 0.5, 1, 1.5, 2, and 4 hours postdose. Additional samples were collected at 6 and 8 hours postdose from women receiving NVP. The maximum plasma concentration (C_{max}) and pre-dose concentration (C_{predose}) were analyzed and the difference between the third trimester and postpartum concentrations were compared.

Adherence data was collected by self-report questionnaire at ante- and post-partum visits.

Statistical Analyses

Plasma HIV RNA concentrations, CD4 counts, and time on ART were compared in a Wilcoxon non-parametric test. The 95% confidence intervals (CI) estimates were calculated by the Modified Wald method. Proportions within ARV arms were compared by Fisher's exact test. *P*-values of ≤ 0.05 were considered statistically significant.

Results

Study population

Sixteen of 38 women enrolled into PACTG 1022 were eligible and evaluated in this substudy (Figure 1); eight women had been randomized to NFV and eight to NVP. The pre-ART plasma HIV RNA and CD4+ cell concentrations were similar between the two arms

(mean 402 log₁₀ c/mL and 322 cells/uL, respectively). Women randomized to NFV received ART an average of 88 days prior to delivery and 236 days postpartum (range: 7–145 and 121–514 days, respectively), which was similar to women randomized to NVP (mean days ART antepartum 125, range: 94–159, $P=0.07$; and mean days postpartum 372, range 85–708 days, $P=0.33$). The substudy participants were infected with HIV-1 subtype B, except one infected with subtype CRF02_AG.

By design all substudy women were ART-naïve and had ART-associated suppression of viral replication. Subsequently, each woman had HIV RNA rebound to >400copies/mL, which in 8 women occurred postpartum in association with discontinuing ART. Importantly, rebound to >400c/mL was detected during gestation and prior to discontinuation of ART in 8 women, suggestive of inadequate antiretroviral (ARV) concentrations due to suboptimal pharmacokinetics, poor adherence, or resistance in the woman's virus population pre-ART. The median pre-ART plasma HIV RNA concentrations (3.85 vs. 3.80 log₁₀ c/mL, $P=0.39$) and median CD4 counts (241 vs. 414 cells/uL, $P=0.09$) did not differ between women with viral rebound during versus after stopping ART postpartum.

HIV drug resistance detected during pregnancy or with discontinuation of ART (Figure 2)

A high rate of drug resistance mutations (11/16, 69%; 95% CI 41–89) was detected by OLA. Consensus sequencing of plasma HIV detected resistance in fewer, 8 of 16 (50%; 95% CI 25–75), consistent with its lower sensitivity.¹⁰ While study records show that the women did not report receiving ARV prior to study entry, two had HIV resistance detected in the enrollment specimen, one in each study arm. The woman assigned to NFV (Participant #10, Figure 2), had mutations conferring resistance to two classes of ARV at enrollment (L90M and K70R) and selected M184V during study ART. The L90M mutation was estimated at 100% in both her pre-ART plasma and PBMC, highly suggestive of transmitted drug-resistance. She had ART-associated suppression of viral replication, but subsequently her virus rebounded with the L90M and K70R mutations and M184V was newly detected. The woman assigned to NVP (Participant #12, Figure 2), had T215Y by OLA at low concentrations in her enrollment PBMC. The T215 codon was indeterminate in plasma virus by OLA, suggestive of a transitional mutation, which by consensus sequencing was T215D. No additional mutations were detected in her virus during study ART. Excluding the mutations in the pre-ART specimens of these two women, new resistance mutations were detected at the time of virus rebound either during gestation or postpartum in 10 of 16 women (63%; 95% CI 35–85).

The rate of selection of new ARV resistance mutations did not differ by treatment arm, occurring in 6/8 (75%) randomized to NFV and 4/8 (50%) to NVP (Figure 2). However, new NRTI mutations were more common in women randomized to NFV compared to NVP: 6/8 (75%) vs. 1/8 (13%), $P=0.04$ (Table 2). The new NRTI mutations included M184V associated with resistance to 3TC/FTC, and K70R and T215Y associated with resistance to thymidine analogs. M184V was detected in the majority of women randomized to NFV 6/8 (75%, 95%CI 35–97) compared to only 1/8 (13%, 95%CI 0–53) given NVP, $P=0.04$.

New NNRTI-resistance mutations were detected in (4/8) women randomized to NVP-, and in none assigned to NFV-ART, $P=0.08$ (Figure 2). K103N was detected in 3/8 (38%), one also had Y181C, and G190A was detected in a fourth participant. New mutations conferring resistance to PI were detected in 2/8 (25%) participants in the NFV arm (Figure 2). Selection of ≥ 2 new resistance mutations to two classes of ART was detected in 4/8 women, 3/8 assigned to NFV- and 1/8 assigned to NVP-ART.

Time to detection of new drug-resistance mutations

During receipt of study ART, the plasma HIV RNA loads rebounded to >400cp/mL in 8 of the 16 women. New resistance mutations were detected in specimens from 4 of these (3/5 NFV and 1/3 NVP), including Participant #10 with L90M and K70R present at entry and newly detected M184V (Table 2). New resistance mutations were detectable only after study ART was discontinued in 6 of the 11 participants (3 NFV and 3 NVP). The median interval between stopping study ART and resistance testing was 88 days: median 77 days (range 0–140) and 88 days (range 49–133) for the NFV and NVP arms respectively. No resistance mutations were detected at any time point in 5 of the 16 women in the substudy, including 3 from the NVP and 2 from the NFV.

Detection of resistance by consensus sequencing and OLA

Testing to detect drug resistance by consensus sequencing (plasma) and OLA (plasma or PBMC) were compared across a total of 42 specimen dates, including $\geq$ two time-points from 15 of the 16 participants. The detection of mutations at codons 30 and 90 in protease and 70, 103, 181, 184, 190, and 215 in RT were tallied for both consensus sequencing and OLA. The mutations identified in consensus sequences were also detected by OLA. In addition, OLA detected 4 resistance mutations in 4 women that were not detected by sequencing, including the mutations encoding K103N, Y181C, M184V, and K70R. Several mutations associated with drug resistance that were not evaluated by OLA were detected by consensus sequencing, including M41L, V118I, E138A, and L210W.

Detection of resistance in plasma versus PBMC by OLA

Resistance mutations were not consistently detected by OLA in both the PBMC and plasma (Figure 2). Across all specimens tested, detection of resistance at specific codons by OLA was greater in the NFV compared to NVP arm when testing plasma ($n=15/151$ in NFV arm versus $4/126$ in NVP arm; $P=0.03$), but rates were similar in PBMC ($n=15/148$ versus $7/109$, respectively; $P=0.37$ (Fisher's exact test)).

NFV and NVP pharmacokinetics and adherence

Ten of the 16 participants had intensive pharmacokinetic data available: 3 receiving NFV (participants 3, 4, 5) and 7 receiving NVP (all participants except 13). The NFV “trough” concentration (C_{predose}) was $0.2 \mu\text{g/mL}$ (range $0.0\text{--}0.5$) in the third trimester and the “peak” (C_{max}) was $2.1 \mu\text{g/mL}$ (range $0.9\text{--}3.7$). The 4-hour postdose value is missing for one of these women which may artificially lower the C_{max} average. The corresponding postpartum values are: C_{predose} and C_{max} were $2.0 \mu\text{g/mL}$ (range $0.4\text{--}3.6$) and $3.8 \mu\text{g/mL}$ (range $2.4\text{--}5.2$) respectively, but were only available for 2 of the 3 women. No second trimester values were available from the women given NFV.

The C_{predose} for the NVP participants in the second trimester was $2.3 \mu\text{g/mL}$ (range $1.6\text{--}3.1$) and C_{max} was $4.4 \mu\text{g/mL}$ (range $3.9\text{--}5.3$). Third trimester values were available for only one woman with C_{predose} and C_{max} of $4.8 \mu\text{g/mL}$ and $6.9 \mu\text{g/mL}$, respectively. Five NVP participants had postpartum values with C_{predose} of $5.7 \mu\text{g/mL}$ (range $3.7\text{--}9.0$) and C_{max} $6.7 \mu\text{g/mL}$ (range $6.0\text{--}8.9$).

Ante- and post-partum adherence data for 11 of the 16 participants indicated missed doses within 30 days of completing the questionnaire. Of the 5 women in the NFV group and 6 women in the NVP group that reported missing doses, only 3 women from each group developed new drug resistance mutations. Three women on NFV and 2 on NVP that reported never missing a dose still developed new resistance mutations.

Discussion

In our study of HIV infected women who took ART for pMTCT, we observed relatively high rates of new HIV drug-resistance mutations. While these women all had ART induced suppression of viral replication, resistance was detected when plasma HIV RNA was >400c/mL during gestation or in viral rebound specimens when ART was stopped between 2 and 24 months postpartum. In this small study, resistance rates did not differ in women randomized to NVP- versus NFV-ART. However, the pattern of resistance differed, with significantly greater selection of resistance to lamivudine (M184V) in women randomly assigned to NFV, and selection of nevirapine resistance (K103N, Y181C and/or G190A) in women randomized to NVP.

The high rate of resistance in our study is consistent with rates reported in a larger observational study of NFV-ART, which detected M184V in 29% by consensus sequencing and 52% by allele-specific PCR.⁵ Mutations encoding M184I/V have been reported across studies of pregnant and postpartum women.^{5,12,13} However, the clinical significance of M184V on later ART has not been evaluated.

When ART is discontinued unequal drug pressure can occur due to variation in the pharmacokinetics of each ARV. The longer half-life of NVP¹⁴ compared to 3TC¹⁵ likely protected participants randomized to NVP-ART from selection of M184V. Instead, when NVP pressure was unopposed NNRTI mutations were preferentially selected. Resistance to NVP and 3TC can occur rapidly because the single base changes that confer high-level resistance likely exist in the viral population due to the permissive nature of HIV polymerase, and when ARV concentrations are low or not complemented by other ARV these mutants are rapidly selected. In contrast, two or more mutations are required for high-level resistance to ZDV and to most PI, except NFV, and the likelihood for two or more specific mutations occurring randomly in the same virus is exceedingly low,⁸ providing a greater genetic barrier to the selection of drug resistant virus.

Several factors, in addition to stopping ART postpartum, could have contributed to the high rates of ARV-resistance detected in this study. First, women who stopped ART postpartum may not have been committed to treatment, and may have had poorer adherence during gestation.¹⁶ Our adherence data showed that half the women in the study reported decreased adherence during gestation, but resistant viruses were selected in women who reportedly adhered to ART during gestation. Second, variable pharmacokinetics of NFV in pregnant women were evident in the women we studied, consistent with previously published studies of both the “older” and “newer” NFV formulations that observed lower and variable third trimester NFV concentrations^{17–22} compared to postpartum values. Low NFV concentrations¹⁷ along with diminished concentrations of the active form of ZDV in a subset of infected cells,²³ would allow selection of 3TC resistance (i.e. low NFV trough concentrations plus low triphosphorylation of ZDV in cells would result in unopposed 3TC pressure and favor the selection of M184V).

The more frequent detection of resistance mutations by OLA compared to consensus sequencing indicates that mutants were at low concentrations in the population.¹⁰ Cell-free virions in the plasma are known to turn-over rapidly, with resistance fading from the plasma within weeks of stopping therapy.^{24,25} The detection of mutants at high concentration in the PBMC and plasma of one participant at the time of study enrollment is consistent with transmitted drug-resistance.²⁶

Our study has several important limitations. The study population was small, and while a significant difference was detected in the selection of M184V, the small group sizes precluded an analysis of differences in the selection of resistance to other ARV. The

increased detection of resistance in the NFV compared to NVP arm when testing plasma by OLA may be biased by the lack of paired specimens for all time-points tested. The absence of ARV concentrations, and information concerning ART adherence practices did not allow us to evaluate the potential mechanisms leading to viral rebound and selection of resistance during pregnancy. Importantly, we were not able to determine whether the resistance first detected in the postpartum specimens of certain women was due to stopping ART or if these women had low ARV concentration throughout gestation due to suboptimal pharmacokinetics or non-adherence. In addition, our study did not evaluate the clinical significance of the resistance on the efficacy of future ART.

Many HIV-infected pregnant women do not qualify for ART based on symptoms or CD4 cell counts. Recently, the WHO revised its pMTCT Guidelines to favor ART regardless of maternal CD4.²⁷ This may increase episodic use of ART during gestation. Comparative studies are needed to select the safest ART regimen for episodic use in pregnancy. Ideally, these studies would compare various gestational ART regimens on the efficacy of subsequent ART. As drug resistant viruses are rarely detected at “virologic failure” of ritonavir-boosted PI regimens,²⁸ either PI-based regimens, or ARV “tails”^{29,30} may diminish the selection of drug-resistant mutants. Given that the detection of mutants in association with ARV given to pMTCT ARV diminish the efficacy of later ART,^{31–33} pre-ART resistance testing could help guide health care workers select ARV with a high likelihood of suppressing viral replication.

In summary, a high rate of drug resistant HIV was detected in women taking NFV- or NVP-based-ART for pMTCT who stopped ART within two years postpartum. 3TC-resistance was selected at a higher rate in women randomized to NFV-ART, which suggests suboptimal NFV pharmacokinetics in pregnancy. As more programs utilize ART for pMTCT, studies such as the NIH-sponsored PROMISE trial are important to inform us on the risks of stopping compared to continuing ART postpartum. Additionally, studies are needed to define the risks of discontinuing various gestational ART regimens on the clinical outcome of ART subsequently administered to treat the mother’s HIV-associated immunodeficiency.

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Figure 1. Schema of P1022 Participant Randomization and Response to ARV

Shading indicates women that were not evaluated for HIV drug-resistance as part of this substudy.

Figure 2. Detection of Drug Resistance by OLA and Consensus Sequencing

□ Specimen not available for assay; ■ Mutant and wild-type genotypes detected by OLA; ■ Mutant genotype detected by OLA; □ WT genotype detected by OLA; □ Indeterminate reaction by OLA; P: Plasma; C: Peripheral Blood Mononuclear Cells; (+) Mutation detected by consensus sequencing; (–) Mutation not detected by consensus sequencing; (*) Mutations assessed only by consensus sequencing.