# Low-Frequency Nevirapine Resistance at Multiple Sites May Predict Treatment Failure in Infants on Nevirapine-Based Treatment

*Dara A. Lehman, MHS, PhD,* Dalton C. Wamalwa, MBChB, MMed, MPH,† Connor O. McCoy, BA,‡ Frederick A. Matsen, PhD,‡ Agnes Langat, MBChB, MMed,† Bhavna H. Chohan, PhD,§|| Sarah Benki-Nugent, PhD,|| Rebecca Custers-Allen, BA,¶ Frederic D. Bushman, PhD,¶ Grace C. John-Stewart, MD, MPH, PhD,|| and Julie Overbaugh, PhD**

**Background:** Resistance commonly arises in infants exposed to single-dose nevirapine (sdNVP) for prevention of mother to child transmission. Although K103N and Y181C are common following sdNVP, multiple other mutations also confer NVP resistance. It remains unclear whether specific NVP resistance mutations or combinations of mutations predict virologic failure in infants when present at low frequencies before NVP-based treatment.

**Methods:** Twenty sdNVP-exposed infants who were subsequently treated with NVP-based highly active antiretroviral therapy were examined. Pretreatment plasma samples were tested for the presence of NVP resistance mutations by allele-specific polymerase chain reaction for K103N and Y181C and ultradeep pyrosequencing (UDPS) for all primary NVP mutations. Viral levels were determined every 3 months for up to 24 months on NVP–highly active antiretroviral therapy. Cox proportional hazard models were used to determine correlates of viral failure.

**Results:** The NVP resistance mutations K103N or Y181C were detected in pretreatment plasma samples in 6 infants by allele-specific polymerase chain reaction. NVP resistance at these or other sites was detectable by UDPS in 10 of 20 infants tested. Virologic failure occurred in 50% of infants with any NVP resistance mutations detected, whereas only 20% of infants without resistance experienced viral failure, but the difference was not significant ($P = 0.19$). An increase in the number of NVP resistance mutations detectable by UDPS in an infant was significantly associated with an increased risk of virologic failure [HR = 1.79 (95% confidence interval: 1.07 to 2.99), $P = 0.027$].

**Conclusions:** Low frequencies of multiple NVP resistance mutations, in addition to K103N and Y181C, present in infants before NVP-based treatment may predict treatment outcome.

**Key Words:** HIV, infants, nevirapine, resistance, HAART, treatment failure

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## INTRODUCTION

A single-dose of nevirapine (sdNVP), a nonnucleoside reverse transcriptase inhibitor (NNRTI) with a relatively long half-life, can reduce HIV-1 mother-to-child transmission by ~50%.[1–3] This simple and effective intervention is an important component of HIV-1 prevention methods in resource-limited settings.[4,5] Unfortunately, the slow decay of NVP often causes drug resistance to develop in both sdNVP-exposed HIV-positive mothers and their infants who become infected despite its use.[6–9] NVP resistance is most often caused by amino acid changes K103N and Y181C in reverse transcriptase (RT) and is typically measured using population-based sequencing assays that detect mutations that comprise >20% of an individual's viral population. Recent studies have shown that after sdNVP, virologic failure during NVP-based highly active antiretroviral therapy (HAART) is increased in both women and infants, even when resistance is undetectable by standard population sequencing.[10,11] These findings suggest that the current standard HIV-1 sequencing assays are not sensitive enough to detect all clinically meaningful resistance.

Because NVP-resistant virus can persist as low-frequency variants for years after sdNVP[12,13] and may compromise responses to NVP-containing treatment, current World Health Organization (WHO) guidelines recommend protease inhibitor (PI)-based HAART in children with any previous NVP exposure, regardless of standard HIV-1 sequencing assay results.[14] However, PI-HAART is not always available as a first-line pediatric option and limits future treatment options if resistance

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From the *Division of Human Biology, Fred Hutchinson Cancer Research Center, Seattle, WA; †Department of Pediatrics, University of Nairobi, Nairobi, Kenya; ‡Program in Computational Biology, Division of Public Health Sciences, Fred Hutchinson Cancer Research Center, Seattle, WA; §Department of Medical Microbiology, University of Nairobi, Nairobi, Kenya; ||Department of Medicine, University of Washington, Seattle, WA; and ¶Department of Microbiology, University of Pennsylvania, Philadelphia, PA.

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Correspondence to: Julie Overbaugh, PhD, Division of Human Biology, Fred Hutchinson Cancer Research Center, 1100 Fairview Ave N, Mailstop C3-168, Seattle, WA 98109-1024 (e-mail: joverbau@fhcrc.org).

arises, as good alternatives are not available in resource-limited settings. Ideally, it would be desirable to preserve the use of NVP-HAART as a first-line regimen, despite sdNVP exposure if resistance at low frequencies could be ruled out.[15]

The suggestion that low-frequency–resistant variants may have a clinical consequence has inspired the development of high sensitivity resistance assays. These include nucleotide-specific methods such as allele-specific polymerase chain reaction (ASPCR) and ultra-deep pyrosequencing (UDPS) methods such as the 454 GS-FLX Titanium system (Roche, Branford, CT). Although ASPCR is relatively inexpensive and high throughput, the reliability of ASPCR can be compromised by polymorphisms in the primer binding sites, a situation that can contribute to false-positive results.[16,17] In addition, ASPCR detects only 1 mutation at a time and has been developed primarily for select NVP-resistance mutations such as K103N and Y181C. There are other NVP resistance mutations,[18] but whether these are clinically relevant when present at low frequencies has not yet been defined. UDPS allows detection of all mutations within a region of interest but is labor intensive, costly, and requires expertise in computational methods.[16,17]

Studies that have examined low-frequency NVP-resistant variants and their impact on subsequent treatment have predominately relied on ASPCR. The largest study on clinical outcome and low-frequency NVP resistance was in women. The findings suggested that levels of K103N, Y181C, or G190A between 1% and 20% detected by ASPCR, which are missed by standard population sequencing assays, can reduce the efficacy of NVP-based treatment of sdNVP-exposed women.[19] It is difficult to directly extrapolate findings in HIV-1–infected adults to infants because the pharmacokinetics of NVP are different in adults and children, as are viral loads and disease progression.[20–22] In particular, viral loads are typically much higher in children,[21] resulting in more viral replication that could contribute to increased viral diversity. One study of infants that examined K103N and Y181C using ASPCR showed a trend for an association between pre-HAART low-frequency resistance and virologic failure.[23] However, low-frequency NVP resistance was not associated with failure in infants who received PI-HAART first and then switched to NVP-HAART.[24] Thus, at present, the contribution of low frequencies of specific NVP resistance mutations, particularly mutations other than K103N and Y181C, to virologic failure during NVP-HAART is not well understood in children. Here we measure resistance using both ASPCR and UDPS in sdNVP-exposed infants who subsequently received NVP-HAART.

## METHODS

### Study Population and Sample Collection

Participants were from 2 ongoing randomized clinical trials initiated in September 2007 in Nairobi, Kenya (Optimizing Pediatric HAART 03 & 04: NCT00428116). Ethical approval was obtained from the University of Washington and Kenyatta National Hospital Institutional Review Boards. Mother–infant pairs were approached at antenatal and postnatal Nairobi city council clinics after screening for HIV-1. In addition, HIV-infected infants identified after admission to Kenyatta National hospital were recruited. After informed consent, a filter paper blood sample was obtained and tested to determine infant HIV-1 status by HIV-1 DNA polymerase chain reaction (PCR). Inclusion in this laboratory substudy was limited to HIV-positive infants with sdNVP administered either to the infant, their mother, or both, as reported by the caregiver or by clinic record. Two cases (designated in Table 1) had probable NVP exposure that could not be confirmed. Additional eligibility criteria were as follows: (1) caregiver planned to reside in Nairobi for >36 months, and (2) age <12 months. Infants included were those who initiated NVP-HAART: usually zidovudine, lamivudine, plus nevirapine. A subset of the infants included (n = 6) was started on NVP-HAART after standard population sequencing was used to rule out high-frequency NVP resistance. In addition, a group of infants (n = 14) was treated with NVP-HAART before resistance testing and switched to PI-HAART after sdNVP exposure was discovered during record review.

At enrollment, blood was collected for CD4 and viral load testing. Caregivers provided demographic information. Three adherence-counseling sessions were provided to all caregivers before HAART initiation. After HAART initiation, blood samples were collected for HIV-1 viral load testing at month 1, 3, and then every 3 months for up to 2 years.

### Laboratory Assays

HIV-1 DNA filter paper assays were performed in Nairobi using a single-round PCR specific to *pol*, which is >98% sensitive and >98% specific.[25] Plasma was separated from whole blood, aliquoted, and stored at −80°C until use. One aliquot was shipped to Seattle in liquid nitrogen. Plasma HIV-1 RNA levels were determined using the Gen-Probe HIV-1 Viral Load Assay (San Diego, CA), which has been validated for detection of HIV-1 subtypes prevalent in Kenya.[26] Virologic failure was defined by ≥2 viral loads of >1000 copies per milliliter or if the last sample available was >1000 copies per milliliter.

For all resistance assays, viral RNA was extracted from 140 μL of plasma using a QIAmp viral RNA kit (Qiagen, Valencia, CA) according to the manufacturer's instructions. Standard resistance testing at "baseline" (after sdNVP and before NVP-HAART) was performed in Nairobi using an in-house population-based sequencing method as described previously.[27] HIV-1 subtypes were determined from the *pol* sequences with PAUP* version 4.0 beta 10 by creating a neighbor-joining phylogenetic tree with reference sequences from the Los Alamos National Laboratory HIV Database (http://www.hiv.lanl.gov/).

### ASPCR to Detect K103N and Y181C

ASPCR to detect K103N and Y181C in pre-HAART plasma samples was performed as described previously[27] with modifications noted here. HIV-1 RNA was normalized to 10,000 RNA copies per 5 μL as defined by the Gen-Probe assay results (targeting >2000 RNA copies per 5 μL assuming

**TABLE 1.** Baseline Characteristics

| ID | Age at NVP-HAART Initiation | Age at Change to PI-HAART | Months of Follow-Up on NVP-HAART | Baseline VL (log Copies/mL) | HIV Subtype | Population Sequencing | Treatment Outcome |
|---|---|---|---|---|---|---|---|
| 7* | 3.5 | — | 21 | 7.2 | C | WT | VF |
| 9 | 11.5 | Died | 6 | 6.7 | A | WT | VF |
| 11 | 12.6 | 33.6 | 21 | 7.4 | A/C | WT | VF |
| 17 | 4.2 | 9.6 | 3 | 7.2 | D | WT | VF |
| 19 | 3.6 | 8.1 | 3 | 5.4 | A | WT | VF |
| 22 | 3 | 15.6 | 12 | 6.2 | A | K103KN | VF |
| 24 | 2.1 | 14.1 | 12 | 6.6 | C/D | WT | VF |
| 2† | 4.8 | — | 24 | 6.0 | A | WT | int |
| 3 | 7 | 21 | 12 | 6.5 | A/C | WT | nf |
| 4 | 2.4 | 10.5 | 6 | 6.6 | A | WT | nf |
| 8‡ | 4.6 | — | 21 | 6.9 | A | WT | nf |
| 12 | 4.8 | 9.9 | 3 | 7.3 | A | K103N | nf |
| 14 | 4.2 | 8.1 | 3 | 7.4 | A | G190A | nf |
| 15 | 2.4 | 7.5 | 3 | 6.0 | A | K103N | nf |
| 18 | 7.4 | 20.4 | 12 | 6.4 | A | WT | nf |
| 20 | 5.1 | 9.9 | 3 | 6.3 | D | WT | nf |
| 28 | 4.5 | 8.1 | 3 | 6.9 | C | WT | nf |
| 29 | 7 | 17 | 9 | 5.4 | A | WT | nf |
| 30 | 6.9 | 14.9 | 6 | 6.1 | A/D | WT | nf |
| 32 | 5.1 | 9 | 3 | 7.1 | A | WT | nf |
| Median | 4.7 | 10.2 | 6 | 6.6 | — | — | — |

*This mother reported NVP-HAART before pregnancy.
†sdNVP exposure unkown due to maternal death before enrollment.
‡Conflicting data on sdNVP exposure.
VL, viral load, age in months, VF, viral failure, int, intermittent viremia, nf, no failure.

an average 20% recovery after RNA extraction and cDNA synthesis efficiency as shown in similar studies)[28] and 5 μL was added to each of duplicate reverse transcriptase–polymerase chain reaction (RT-PCRs). All PCR conditions and primer sequences are described in Supplemental Digital Content 1 (see **Table S1**, http://links.lww.com/QAI/A301). To minimize errors in quantification due to polymorphisms in the ASPCR primer binding sites, a "cure" step, which uses a low annealing temperature and long primer to incorporate an invariant sequence proximal to the mutation site,[29] was performed following RT-PCR and before ASPCR (Teri Liegler, PhD, personal communication, May 2010). The RT-PCR product was diluted 1:1000, and 5 μL were added to the cure step. The cure product was then diluted 1:50 and 5 μL added to parallel reactions of allele-specific or total copy real-time PCR performed on an ABI prism 7900HT.

To determine the error rate and lower limit of detection of ASPCR, controls were performed with mixtures of wild type and K103N/Y181C-containing HIV-1 in vitro transcripts. RNA was in vitro transcribed from plasmids containing the *pol* region of Q23-17, a subtype A HIV-1 clone,[30] and quantified by spectrophotometer before diluting and mixing in estimated ratios of 100%, 10%, 5%, 3%, 1.2%, 0.4%, and 0% mutant. ASPCR results from these mixtures were used in a receiver operator characteristic analysis and the cut-off with 100% specificity and maximum sensitivity (90.9% and 91.7% for K103N and Y181C, respectively) was used to define the lower limit.

Samples were run in duplicate and gel electrophoresis was performed on the ASPCR product. 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 to zero. The proportion of drug-resistant virus was calculated for each reaction, and if it was below the lower limit or the correct size band was not visible on the gel, the proportion for that replicate was set to zero. The average proportion of drug-resistant to wild type was calculated by averaging the replicate results from each sample.

## UDPS Using the 454 GS-FLX Titanium System

We performed 10 parallel high fidelity RT-PCR reactions in the pre-HAART plasma samples, each with 10,000 viral copies based on the viral load data. Second-round PCR to amplify a 555bp product spanning RT codons 50–234 was performed with primers linked to 454 sequencing adapters and a unique 8bp barcode (see **Table S1, Supplemental Digital Content 1**, http://links.lww.com/QAI/A301). The 10 parallel PCR products from each sample were pooled, gel isolated, and the resulting DNA concentration determined using the Qubit dsDNA HS kit (Invitrogen). The barcoded PCR amplicons were then mixed in equimolar concentrations and pyrosequenced in both forward and reverse directions on the 454 GS-FLX titanium platform at the University of Pennsylvania.

## UDPS Data Processing

The UDPS sequences were grouped by sample according to an exact barcode-primer match. A quality-filtering step truncated the sequences at the first ambiguous base or when the mean quality score in a 50bp sliding window dropped below 25. Sequences <100bp after quality filtering were discarded. Primers were trimmed before analysis.

Cleaned sequences were pair-wise aligned to a subject-specific population-based sequence using the Needleman–Wunch algorithm (gap opening penalty: 20.0, gap extension penalty: 0.5, EDNA 4.4 scoring matrix).[31] Deletions relative to the subject-specific reference sequence at a resistance site were treated as missing data. Insertions were accommodated by adding gaps to preserve the alignment, which was then confirmed by visual inspection near sites of resistance. Each sample was evaluated for the presence of any NVP resistance mutations that confer at least moderate resistance (score ≥30) according to the Stanford resistance database.[18] Forward sequence reads were used to assess RT amino acids 100–106, and reverse reads were used for amino acids 151–215. Resistance sites were located on the reference population-based sequence, and the aligned UDPS sequences were categorized as wild type or mutant at each resistance site by a computer program developed in-house. Alignments and mutant categories were confirmed by visual inspection.

A Fisher exact test was used to test samples for elevated mutation rates relative to false-positive rates in the pooled results of nonmutant controls. The *P* values were Bonferroni corrected (alpha = 0.10). A lower limit of detection was determined for the minimum, median, and maximum read count at each position examined (see **Table S2, Supplemental Digital Content 2**, http://links.lww.com/QAI/A302) by testing increasing mutation counts from zero until the Bonferroni-corrected *P* value was statistically significant.

## Statistical Analysis

Kaplan–Meier survival curves and Cox proportional hazards models with robust standard errors were used to determine pre-HAART correlates of virologic failure. Infants without viral failure were censored at switch to PI-HAART or after 21–24 months of follow-up. In the Cox models, changes to different amino acids at the same position were counted as a single site of resistance. All analyses were performed using Stata version 9.2 (College Station, TX).

# RESULTS

## Study Population

The study population included 20 infants who had been exposed to sdNVP, directly through administration to their mothers during labor, or both, and who were subsequently treated with NVP-HAART. The infants ranged in age from 2.1 to 12.6 months at HAART initiation (Table 1). The median pre-HAART baseline viral load was 6.6 $\log_{10}$ copies per milliliter. Phylogenetic analysis indicates that 60% of infants were infected with subtype A, 10% with subtype C, 10% with subtype D, and 20% with intersubtype recombinants.

Four infants had resistance detectable by population-based sequencing before HAART initiation as follows: 3 had the K103N mutation and 1 had G190A, both of which confer high levels of NVP resistance (Table 1). No other NNRTI or nucleoside reverse transcriptase inhibitor (NRTI) resistance mutations were detectable by population sequencing in these pre-HAART samples. Seven infants experienced virologic failure while on NVP-HAART, 1 infant had intermittent viremia in which viral levels rose above 1000 copies per milliliter but then dropped below detection on subsequent measures (ID# 2) and was not categorized as virologic failure, whereas 12 maintained virologic suppression throughout follow-up (Table 1).

## Detection Limits of High-Sensitivity ASPCR and UDPS Resistance Testing

To compare the sensitivity of our high sensitivity resistance assays, we performed ASPCR and UDPS on duplicate low-frequency mixtures of RNA transcripts ranging from 0.1% to 10% K103N/Y181C and 0% and 100% mutant controls. Both ASPCR and UDPS were able to quantify K103N and Y181C in mixtures with as little as 0.4% mutant at levels above the error rate seen in the 0% controls (Table 2). The quantification of all the low-frequency mixtures was consistently 2-fold to 5-fold below the expected mutant frequencies. Given the consistency across samples and using both methods, this difference was likely due to inaccuracies in the initial quantification of the in vitro transcripts used and/or pipetting during the mixture preparations. Data from the 0% mutant controls reveal the error rates (false positives) at K103N and Y181C, which ranged from 0.05% to 0.55% depending on the assay used (Table 2). These error rates were used to define the lower limits of detection for the 2 assays as follows: 0.97% and 0.54% for K103N and Y181C by ASPCR, and ranged from 0.16%–0.27% and 0.26%–0.37% for K103N and Y181C by UDPS, which varies depending on the number of sequence reads.

UDPS detection limits at other sites of resistance, shown in Supplemental Digital Content 2 (see **Table S2,** http://links.lww.com/QAI/A302), vary both by site and sequence depth and are comparable with those published in

**TABLE 2.** ASPCR and UDPS on Low-Frequency K103N/Y181C Controls

| | % K103N Controls | | % Y181C Controls | |
|---|---|---|---|---|
| **Expected** | **ASPCR** | **UDPS** | **ASPCR** | **UDPS** |
| 100% | 94.70% | 99.86% | 95.40% | 99.94% |
| 10% | 6.55% | nt | 4.85% | nt |
| 5% | 2.95% | 1.08% | 1.55% | 1.08% |
| 3% | 1.53% | nt | 1.01% | nt |
| 1.2% | 0.95% | 0.22% | 1.07% | 0.27% |
| 0.4% | 0.98% | 0.18% | 0.47% | 0.25% |
| 0.1% | nt | 0.03% | nt | 0.06% |
| 0% | 0.55% | 0.05% | 0.16% | 0.07% |
| Lower limit | 0.97% | 0.16%–0.27% | 0.54% | 0.26%–0.37% |

nt, not tested.

studies using similar methods.[28] These detection limits were derived from the false-positive error rates of the UDPS data on the RNA transcripts (wild type at these positions), which varied from 0.01% to 2.55% (see **Table S3, Supplemental Digital Content 3**, http://links.lww.com/QAI/A303).

## Frequency of K103N and Y181C by ASPCR in Pre-HAART Infant Plasma

K103N was detected by ASPCR in 4 infants at levels ranging from 18.7% to ~100% and Y181C in 3 infants at 0.9%–3.1% (Table 3). The 3 infants in which K103N was detected by population sequencing (ID# 12, 15, 22) had K103N levels >20% as quantified by ASPCR. In addition, ASPCR detected K103N in 1 infant and Y181C in 3 infants at <20% (Table 3). In 1 case, both K103N and Y181C were detectable by ASPCR, whereas in 5 cases only 1 or the other mutation was detected (Table 3).

## Additional Low-Frequency NVP-Resistant Variants Detected by UDPS

Resistance mutations K103N and Y181C were detected by UDPS in 5 and 2 infants, respectively (Table 3). The level of K103N by UDPS was within 2-fold of that quantified by ASPCR in samples with >10% K103N. For example, levels of K103N were 18.7% versus 10.6% (ID# 24), and 63.5% versus 99.7% (ID# 15) by each method (Table 3). Quantification below 10% was less concordant between assays. Only 1 sample had low levels of Y181C measured by both assays (#22), whereas 4 low-frequency measurements were discordant between UDPS and ASPCR, 2 each detected by 1 method and not the other (Table 3).

**TABLE 3.** K103N and Y181C Detected by ASPCR Versus UDPS

| | ASPCR | | UDPS | |
|---|---|---|---|---|
| ID | %K103N | %Y181C | %K103N | % Y181C |
| 7 | bd | 0.9% | bd | bd |
| 9 | bd | bd | bd | bd |
| 11 | bd | bd | bd | bd |
| 17 | bd | bd | bd | bd |
| 19 | bd | 1.0% | bd | bd |
| 22 | 48.9% | 3.1% | 49.90% | 0.49% |
| 24 | 18.7% | bd | 10.62% | bd |
| 2 | bd | bd | bd | bd |
| 3 | bd | bd | bd | bd |
| 4 | bd | bd | bd | bd |
| 8 | bd | bd | bd | bd |
| 12 | 106.0% | bd | 99.83% | bd |
| 14 | bd | bd | bd | bd |
| 15 | 63.5% | bd | 99.71% | bd |
| 18 | bd | bd | bd | bd |
| 20 | bd | bd | 0.18% | 0.36% |
| 28 | bd | bd | bd | bd |
| 29 | bd | bd | bd | bd |
| 30 | bd | bd | bd | bd |
| 32 | bd | bd | bd | bd |

bd, below detection.

We analyzed the UDPS data at other sites known to cause intermediate to high-level NVP resistance.[18] G190A was present at a high frequency (>20%) in 1 infant (#14), which is consistent with results of population sequencing. Other resistance mutations detected by UDPS, but not by population sequencing, include K101E, K103S/T, V106A/M, G190T, and F227C and ranged from 0.06% to 9.97% (Table 4). Mutations that were assayed by UDPS but did not have detectable levels in any samples tested included L100I, K101P, V179F, Y181I/S/V, Y188C/H/L, and G190C/Q/S/V (data not shown). The most common mutation detected was K103N in 5 infants, followed by G190A in 4 of 20 infants. Of the 10 infants who had detectable resistance by UDPS, 4 had resistance at more than 1 site (Table 4).

In addition, we analyzed the UDPS data at RT amino acids 151, 184, and 215 known to confer high-level resistance to NRTIs. Only M184I was present at detectable levels as follows: 0.13% and 0.23% in infants 9 and 17, respectively (Table 4).

## Number of Sites With NVP Resistance is Associated With Virologic Failure

We used a Cox proportional hazards model to determine the predictors of virologic failure (Fig. 1A). First, we tested whether the presence, before HAART, of the 2 most common high-level NVP resistance mutations, K103N and Y181C, were associated with failure during NVP-HAART. Among the 5 infants that had detectable levels of K103N or Y181C by UDPS, 40% experienced virologic failure whereas failure was observed in 33% of infants without detectable K103N/Y181C, but the difference was not statistically significant {HR = 1.90 [95% confidence interval (CI): 0.51 to 7.12]; $P = 0.34$}. When we included the presence of K103N or Y181C detected by any method, there was a trend toward an association [HR = 5.21 (95% CI: 0.94 to 28.93) $P = 0.059$].

Next, we tested whether the presence of any NVP resistance mutation at any level was associated with failure. Virologic failure occurred in 50% of infants with any NVP resistance mutations detected, whereas only 20% of infants without resistance experienced viral failure. This difference is shown in the Kaplan–Meier curve in Figure 1B, but the difference seen here did not reach statistical significance [HR = 3.06 (95% CI: 0.57 to 16.46); $P = 0.19$; Fig. 1A]. A significant association was observed between the number of sites with NVP resistance detectable by UDPS and virologic failure [HR = 1.79 (95% CI: 1.07 to 2.99); $P = 0.027$], suggesting an increased risk of failure with each additional site of resistance. Furthermore, when we included both NNRTI and NRTI mutations, the association between the number of sites with resistance detectable by UDPS and viral failure was highly significant [HR = 2.21 (95% CI: 1.41 to 3.48), $P = 0.001$; Fig. 1A]. In addition, the infants with a sum of more than 1% of viruses with any resistance mutations, which is a predictor of viral failure in adults,[19] had a trend toward an increased risk of viral failure compared with infants with <1% resistance [HR = 3.40 (95% CI: 0.84 to 13.80); $P = 0.087$]. When we excluded infants with high-frequency resistance

**TABLE 4.** NVP Resistance by UDPS in pre-HAART Infant Plasma

| ID | Minimum No. 454 Reads | NVP Resistance Mutations Detected by UDPS* | | | | | | | | | | |
|---|---|---|---|---|---|---|---|---|---|---|---|---|
| | | K101 | K103 | | | V106 | | Y181 | G190 | | | F227 |
| | | E | N | S | T | A | M | C | A | E | T | C |
| 7 | 11134 | 0.21% | bd | bd | bd | bd | bd | bd | bd | bd | bd | bd |
| 9 | 8598 | bd | bd | bd | bd | bd | bd | bd | bd | bd | bd | bd |
| 11 | 12999 | bd | bd | bd | bd | bd | bd | bd | bd | bd | bd | 0.07% |
| 17 | 11029 | bd | bd | bd | bd | bd | bd | bd | bd | bd | bd | bd |
| 19 | 6203 | 0.20% | bd | bd | bd | bd | bd | bd | 3.76% | bd | bd | bd |
| 22 | 9529 | bd | 49.90% | bd | 0.32% | bd | bd | 0.49% | 0.34% | bd | bd | bd |
| 24 | 4212 | bd | 10.62% | bd | 0.80% | 9.97% | 0.19% | bd | 0.65% | bd | bd | bd |
| 2 | 5374 | bd | bd | bd | bd | bd | bd | bd | bd | bd | bd | bd |
| 3 | 15058 | bd | bd | bd | bd | bd | bd | bd | bd | bd | bd | bd |
| 4 | 14402 | bd | bd | bd | bd | bd | bd | bd | bd | bd | bd | bd |
| 8 | 10382 | bd | bd | bd | bd | bd | bd | bd | bd | bd | bd | bd |
| 12 | 14318 | bd | 99.83% | 0.18% | bd | bd | bd | bd | bd | bd | bd | bd |
| 14 | 4497 | bd | bd | bd | bd | bd | bd | bd | 99.95% | bd | 0.06% | bd |
| 15 | 13633 | bd | 99.71% | 0.13% | bd | bd | bd | bd | bd | bd | bd | bd |
| 18 | 15305 | bd | bd | bd | bd | bd | bd | bd | bd | 0.64% | bd | bd |
| 20 | 14478 | bd | 0.18% | bd | bd | bd | bd | 0.36% | bd | bd | bd | bd |
| 28 | 13964 | bd | bd | bd | bd | bd | bd | bd | bd | bd | bd | bd |
| 29 | 4923 | bd | bd | bd | bd | bd | bd | bd | bd | bd | bd | bd |
| 30 | 7783 | bd | bd | bd | bd | bd | bd | bd | bd | bd | bd | bd |
| 32 | 13646 | bd | bd | bd | bd | bd | bd | bd | bd | bd | bd | bd |

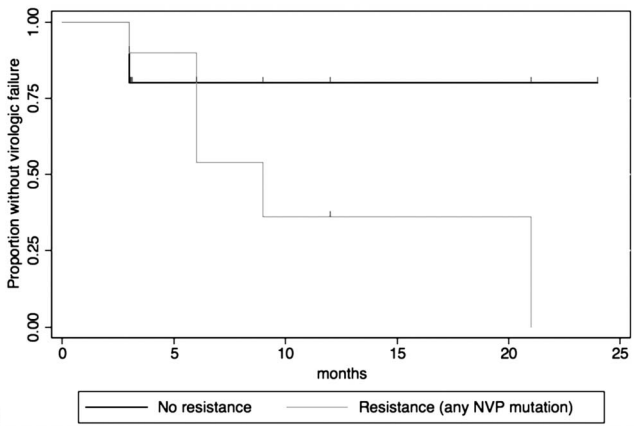
| ID | NRTI† M184 I | Total % Resistance | No. Sites With NVP Resistance‡ | No. Sites NVP + NRTI Resistance‡ | Months of Follow-Up | Time of VF |
|---|---|---|---|---|---|---|
| 7 | bd | 0.21% | 1 | 1 | 21 | Month 6 |
| 9 | 0.13% | 0.13%§ | 0 | 1 | 6 | Month 3 |
| 11 | bd | 0.07% | 1 | 1 | 21 | Month 21 |
| 17 | 0.23% | 0.23%§ | 0 | 1 | 3 | Month 3 |
| 19 | bd | 3.96% | 2 | 2 | 3 | Month 3 |
| 22 | bd | 51.05% | 3 | 3 | 12 | Month 6 |
| 24 | bd | 22.24% | 3 | 3 | 12 | Month 9 |
| 2 | bd | 0% | 0 | 0 | 24 | int |
| 3 | bd | 0% | 0 | 0 | 12 | nf |
| 4 | bd | 0% | 0 | 0 | 6 | nf |
| 8 | bd | 0% | 0 | 0 | 21 | nf |
| 12 | bd | 100.01% | 1 | 1 | 3 | nf |
| 14 | bd | 100.01% | 1 | 1 | 3 | nf |
| 15 | bd | 99.83% | 1 | 1 | 3 | nf |
| 18 | bd | 0.64% | 1 | 1 | 12 | nf |
| 20 | bd | 0.54% | 2 | 2 | 3 | nf |
| 28 | bd | 0% | 0 | 0 | 3 | nf |
| 29 | bd | 0% | 0 | 0 | 9 | nf |
| 30 | bd | 0% | 0 | 0 | 6 | nf |
| 32 | bd | 0% | 0 | 0 | 3 | nf |

*NVP resistance mutations assayed by UDPS with no detectable resistance in any samples are not included in table: L100I, K101P, V179F, Y181I/S/V, Y188C/H/L, G190C/Q/S/V.
†NRTI resistance mutations assayed by UDPS with no detectable resistance in any samples are not included in table: Q151M, M184V, T215F/Y.
‡Changes to different amino acids at the same position were counted as a single site of resistance.
§For these cases, resistance would be 0% if NRTI sites were excluded.
bd, below detection, VF, viral failure, int, intermittent viremia, nf, no failure.

**Cox hazards model: predictors of virologic failure**

| Covariate | p-value | HR (95% CI) |
|---|---|---|
| K103N/Y181C by UDPS | 0.34 | 1.90 (0.51, 7.12) |
| K103N/Y181C by any method | 0.059 | 5.21 (0.94, 28.93) |
| Any NVP resistance mutation | 0.19 | 3.06 (0.57, 16.46) |
| Number of sites with NVP resistance | 0.027 | 1.79 (1.07, 2.99) |
| Number of sites with NVP or NRTI resistance | 0.001 | 2.21 (1.41, 3.48) |
| NVP or NRTI resistance sum over 1% | 0.087 | 3.40 (0.84, 13.80) |
| Age >6 months | 0.56 | 0.62 (0.12, 3.14) |
| Log viral load | 0.61 | 1.42 (0.36, 5.56) |

A




B




**FIGURE 1.** Predictors of virologic failure. A, Univariate Cox proportional hazards models used to determine associations between pre-HAART characteristics and virologic failure. B, Kaplan–Meier curve of the proportion of infants without virologic failure comparing infants with no detectable pre-HAART NVP resistance by UDPS (black line) to infants with any level of any NVP resistance mutation at baseline (grey line).

detectable by population-based sequencing (Table 1), low-frequency resistance of >1% was significantly associated with viral failure [HR = 4.7 (95% CI: 1.3 to 16.8), $P = 0.016$].

No other baseline characteristics were associated with virologic failure, including age at HAART initiation or log viral load.

## DISCUSSION

A recent randomized trial showed that sdNVP exposure increases the risk of virologic failure in children subsequently treated with NVP-HAART.[10] These results prompted the WHO to recommend that all NVP-exposed children receive PI-HAART.[14] However, reliance on PI-HAART for all NVP-exposed children is problematic as PI-HAART is toxic, heat-labile, and costly, and alternative regimens are limited in resource-poor settings when resistance arises. Therefore, defining the contribution of low-frequency NVP-resistant variants to treatment failure in children may help define the optimal long-term treatment of NVP-exposed infants. Here we utilized 2 highly sensitive assays to detect low-frequency–resistant variants in children subsequently treated with NVP-HAART. We observed an increased risk of virologic failure with increasing numbers of detectable NVP resistance mutations, even when present at low frequencies.

To our knowledge, this is the first study to utilize UDPS to detect multiple NVP resistance mutations in a cohort of NVP-exposed children. The most common mutations detected were K103N and G190A, whereas Y181C was rare. This was surprising given that Y181C is the most common mutation detected in infants in other studies.[7,24,32,33] Although 10 infants in this study had no detectable resistance mutations, 6 had a NVP resistance mutation at a single amino acid position, 2 infants had 2 sites of NVP resistance, and 2 had NVP resistance at 3 sites. An increasing number of sites of resistance was associated with an increased risk of viral failure, suggesting that assays such as UDPS that capture all relevant NVP resistance mutations may provide a more comprehensive view of the role of low-frequency resistance in treatment outcome than assays limited to select changes. We have very few clear cases of NRTI-based resistance, but interestingly, the 2 infants (ID# 9, 17) who experienced viral failure despite a lack of detectable NVP resistance did have an NRTI mutation (M184I) present at low frequency. When we include both the NVP and NRTI resistance data in our analysis, the association between the number of sites with resistance and viral failure was highly significant ($P = 0.001$). Furthermore, of the 10 infants without detectable NVP resistance at any of the sites tested, 8 did not experience virologic failure during follow-up. This further supports the idea that mutations in addition to K103N or Y181C could be used to screen for resistance before NVP-based treatment.

Here we showed that the results of UDPS and ASPCR are concordant when resistance is present at high frequencies. However, there were cases in which low-frequency resistance was detected by one method and not the other, and neither method was clearly superior for detecting K103N or Y181C. The one other study that has compared these assays to quantify low levels of K103N during intermittent NNRTI-based HAART also had some discrepant results when quantifying resistance at low frequencies.[34] Given that ASPCR can produce false-positives in diverse clinical samples[17] and that our control data suggest UDPS is more sensitive than ASPCR (Table 2), the UDPS data may be a more accurate measure of low-frequency resistance.

The limitations of our study include a small sample size and short follow-up on NVP-HAART that resulted from study protocol changes due to the change in the WHO recommendation for treating NVP-exposed children.[10,14] The lack of association seen between K103N or Y181C and viral failure could reflect the small sample size and limited follow-up, as other larger studies have found this association.[19] Six of the 8 infants that were followed for only 3 months are in the no failure group, of which 4 had detectable resistance. Given the short duration of follow-up, it is possible that some of these infants are misclassified. Indeed, 3 infants with high frequencies of resistance were followed on NVP-HAART for only 3 months before switching to PI-HAART and might have experienced viral failure with longer follow-up (ID# 12, 14, 15). This would bias our analysis toward the null

hypothesis that there is no difference in resistance between the 2 groups. Despite these limitations, we detected an association between the number of NVP resistance mutations and NVP-based treatment failure. The limited follow-up may also have contributed to the lack of association between age at HAART initiation (analogous to time since sdNVP) and treatment failure. Other studies suggest that NVP resistance and the clinical consequence of sdNVP fade with time.[11,35,36]

Our results are consistent with other studies that showed that low frequencies of NVP resistance have clinical consequence[16,19,23] and suggest that mutations other than K103N and Y181C may be relevant. Thus, to fully understand the contribution of low-frequency resistance to treatment failure, a comprehensive survey of the potential contributing resistance mutations is needed. Although UDPS is presently not amenable to wide-scale application in resource-limited settings, it provides a potentially useful research tool for understanding the interplay between baseline resistance and treatment outcome. In the longer term, more cost-effective lower technology methods to detect a range of resistance mutations could prove valuable for optimizing HIV treatment.

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