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HIV-1 drug resistance at antiretroviral treatment initiation in children previously exposed to single-dose nevirapine

Gillian M. Hunt[a], Ashraf Coovadia[b], Elaine J. Abrams[c], Gayle Sherman[d], Tammy Meyers[e], Lynn Morris[a] and Louise Kuhn[f]

Objective: To describe the prevalence of HIV-1 drug resistance mutations at the time of treatment initiation in a large cohort of HIV-infected children previously exposed to single-dose nevirapine (sdNVP) for prevention of transmission.

Design: Drug resistance mutations were measured pretreatment in 255 infants and young children under 2 years of age in South Africa exposed to sdNVP and initiating ritonavir-boosted lopinavir-based therapy. Those who achieved viral suppression were randomized to either continue the primary regimen or to switch to a nevirapine-based regimen. Pretreatment samples were tested using population sequencing and real time allele-specific PCR (AS-PCR) to detect Y181C and K103N minority variants. Those with confirmed viremia more than 1000 copies/ml by 52 weeks postrandomization in the switch group were defined as having viral failure.

Results: Nonnucleoside reverse transcriptase inhibitor (NNRTI) mutations, predominantly Y181C, were detected by either method in 62% of infants less than 6 months of age, in 39% of children 6–12 months of age, 22% 12–18 months, and 16% 18–24 months ($P = <0.0001$). NNRTI mutations detected by genotyping, but not K103N or Y181C mutations detected only by AS-PCR, were associated with viral failure in the switch group.

Conclusion: The prevalence of mutations known to compromise primary NNRTI-based therapy is high in sdNVP-exposed children, supporting current guidelines recommending use of protease inhibitor-based regimens for young children. Standard genotyping is adequate to identify children who could benefit from switching to NNRTI-based therapy.
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Keywords: antiretroviral therapy initiation, HIV-1 drug resistance, infants and young children, single-dose nevirapine

Introduction

Single-dose nevirapine (sdNVP) has been widely used in low-resource settings for prevention of mother-to-child HIV transmission (pMTCT) and NVP continues to be used as part of more complex prophylactic and therapeutic regimens [1]. It is now well established that NVP used for pMTCT selects viral mutations associated with resistance to nonnucleoside reverse transcriptase inhibitors (NNRTI) among a large proportion of exposed women [2–7]. Infants who fail prophylaxis and who acquire infection despite NVP exposure also develop resistance. Because

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transmission rates are low with antiretroviral prophylaxis, the number of children included in cohorts is small and infants have been mostly studied only 6–8 weeks after exposure [8–11].

The proportion of HIV-infected infants who have NNRTI mutations 6–8 weeks after sdNVP is usually higher than that observed among women at comparable times after exposure [2,8,11]. A meta-analysis estimated that the prevalence of NNRTI mutations 6 weeks after exposure was 56% [12], although higher rates (87%) have been observed in some subgroups [13]. In contrast to adults in whom K103N predominates, the predominant mutation among infants is Y181C [2,9,11,14]. Persistence of mutations has only been studied in small numbers of infants, but, like in adults, the prevalence of mutations declines with increasing time after exposure [9,15]. Persistence is important as it is the mutations still present at the time of treatment initiation that predict virologic response to NNRTI-based treatment [16,17]. For clinical and public health purposes, it is the prevalence of NNRTI mutations in infants and young children at the time of treatment initiation that needs to be accurately quantified.

It is also well established that standard methods of bulk population sequencing miss drug resistance mutations when they are present at low levels. Several more sensitive assays to detect minority variants have been developed, including real-time allele-specific PCR (AS-PCR), point mutation assays (LigAmp), oligonucleotide ligation assays (OLAs), and pyrosequencing [7,8,11,14,15,18–20]. When these methodologies are used, a larger proportion of children are found to harbor mutations [8,14,15,20]. However, the small numbers of children tested and the limited examination of later time points precludes a confident estimate of the proportion of children whose treatment may be compromised by these selected variants.

Here we examined the prevalence of drug resistance at the time of initiation of ritonavir-boosted lopinavir (LPV/r)-based antiretroviral therapy (ART) in a large cohort of HIV-infected infants and young children in South Africa who had previously received sdNVP as part of pMTCT. We ascertained pretreatment resistance using both standard genotyping and more sensitive AS-PCR methods for the Y181C and K103N mutations. Children who suppressed on the initial regimen were randomized to either continue the primary regimen or to switch to a NVP-based regimen. We further investigated whether pretreatment resistance mutations measured using each of these methods were associated with virological failure.

Methods

Samples from HIV-1-infected children

Samples for this study were collected at baseline of a randomized clinical trial designed to evaluate a novel strategy for preserving NVP as a component of treatment regimens for sdNVP-exposed children [21]. Pretreatment plasma samples were obtained from 255 HIV-1-infected children less than 24 months of age, who were exposed to sdNVP for pMTCT, and who met criteria for and initiated LPV/r-based ART. Children were enrolled at Rahima Moosa Mother and Child Hospital in Johannesburg, South Africa between April 2005 and July 2007. Eligibility criteria for treatment included WHO stage III or IV disease, CD4% less than 25 if younger than 12 months or less than 20 if older than 12 months, or recurrent (>2/year) or prolonged (>4 weeks) hospitalization for HIV-related complications. Pretreatment samples were tested for HIV-1 RNA quantity (Roche Amplicor version 1.5; Roche, Branchburg, New Jersey, USA) and CD4 cell count and percentage. Detailed histories were obtained and neither mothers nor children were reported to have been exposed to antiretroviral drugs other than sdNVP. Overall, 28% of children were ever breastfed and the median duration of breastfeeding in those who initiated any breastfeeding was 60 days. Members of the cohort who achieved viral suppression on the LPV/r-based primary regimen were randomized to either continue the primary regimen or to switch to a NVP-based regimen and were followed with regular measurements of HIV-1 RNA quantity. Those with confirmed viremia more than 1000 copies/ml by 52 weeks postrandomization were defined as having viral failure [21]. Signed informed consent was obtained from the children's caregivers and the study was approved by the Institutional Review Boards of the University of the Witwatersrand and Columbia University.

Genotyping of the polymerase gene

Sequencing of the *pol* gene was done using an in-house assay certified by the Virology Quality Assessment (VQA) Program. Briefly, viral RNA was isolated from plasma using a MagNa Pure LC Total Nucleic Acid Isolation kit on the MagNa Pure Automated System (Roche Diagnostics, Indianapolis, Indiana, USA). A nested PCR was performed as previously described to generate a 1.7 kb amplicon spanning both the protease and reverse transcriptase genes [22]. In cases in which amplification of the *pol* gene was not obtained, the protease and reverse transcriptase regions were amplified separately. The first PCR was performed as previously described to generate a 985 bp amplicon of the reverse transcriptase gene [23], whereas the second nested PCR amplified 490 bp of the polymerase gene spanning the protease gene. The protease PCR used the same conditions as the reverse transcriptase PCR, but differed in that primers polCF (5′ GAAGGACACCAAATGAAAGACTGTAC) and polIRS (5′ ACTCTGGAATATTGCTGGTGATCC) were used for the initial reaction, and protease F (5′ CTTCAGAACAGACCAGAGC 3′) and protease R (5′ CTCTTCTGTTAACGGCCATTG 3′) for the nested reaction. The PCR products were sequenced using BigDye Terminators v3.1 on an ABI3100 Genetic Analyzer

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(Applied Biosystems, Foster City, California, USA). Consensus sequences were aligned and manually edited using the Sequencher v4.5 software (GeneCodes, Ann Arbor, Michigan, USA). Genotypic resistance was defined as the presence of resistance mutations associated with impaired drug susceptibility, using the Stanford Genotypic Resistance Interpretation Algorithm (http://hivdb.stanford.edu/) and the December 2009 International AIDS Society drug resistance mutation list [24]. Phylogenetic analysis of nucleic sequences was performed using MEGA 3.1 for internal quality purposes, and using reference sequences downloaded from Los Alamos (www.lanl.gov).

Allele-specific PCR for K103N and Y181C

PCR products were further tested for the K103N mutation using an AS-PCR assay, as previously described [7,23]. Analysis of synthetic plasmid mixtures and treatment-naive samples showed a detection cut-off of 0.2% for the minor variant. All positive samples were re-tested twice and only those positive on all three repeats were considered true positives.

A second AS-PCR was designed to discriminate between the Y and C alleles at position 181 of the *reverse transcriptase* gene. The reaction was performed on the LightCycler 480, using LightCycler 480 SYBR Green 1 Master (Roche Applied Science, Mannheim, Germany) and primers 71F, 75R (internal control), 76R (TGT), and 77R (TAT) [18]. The primers were added to a final concentration of 300 nmol/l, and the reaction performed in a total volume of 25 μl. Analysis of a dilution series of wild-type 181Y and mutant 181C plasmid mixtures and 80 treatment-naive samples showed the lower limit of detection to be 0.4%.

Statistical analyses

We calculated the proportions of children with NNRTI mutations detected using each method and compared proportions between groups using χ^2 tests. Stratified analyses were done wherever appropriate. Descriptive statistics, including medians and 25–75th percentiles to describe the interquartile range (IQR), were used. The association between pretreatment resistance and viral failure was examined in those randomized to NVP-based therapy and rates of failure compared between groups using χ^2 tests. Data were analyzed using GraphPad InStat v3.06, San Diego, California, USA.

Results

Detection of HIV-1 drug resistance mutations using population sequencing

Population sequencing detected major antiretroviral drug resistance mutations from protease inhibitor, NRTI, and NNRTI drug classes in 31% (80/255) of all infants and children. As expected, NNRTI mutations were most prevalent and were present in 27% (69/255) of samples: 20% had Y181C and 5% K103N (Fig. 1). The G190A and Y188C mutations were each detected in two samples and V106I/M in one sample. NNRTI minor mutations were also detected, specifically E138A ($n=20$), V179D ($n=7$), V90I ($n=3$), and A98G ($n=1$), as well as the NNRTI-associated polymorphisms K101Q/T ($n=3$), E138G/S ($n=3$), V179A ($n=2$), H221Y ($n=2$), and L234P ($n=1$). Nineteen samples had major NRTI mutations, specifically K219E/Q ($n=8$), L74V ($n=7$), K70E ($n=3$), D67N ($n=2$), and L210W ($n=1$). Other NRTI-associated mutations detected included T69A/N/S ($n=21$), V118I ($n=9$), V75L ($n=3$), and T215A/I ($n=3$). The major protease inhibitor mutations M46I/L and I47V were present in four samples, and minor protease inhibitor mutations L10I/V, V11I, A71T, I85V, and N88D were also detected. The protease inhibitor mutation T74S was found in 29 samples, and one sample had an insertion

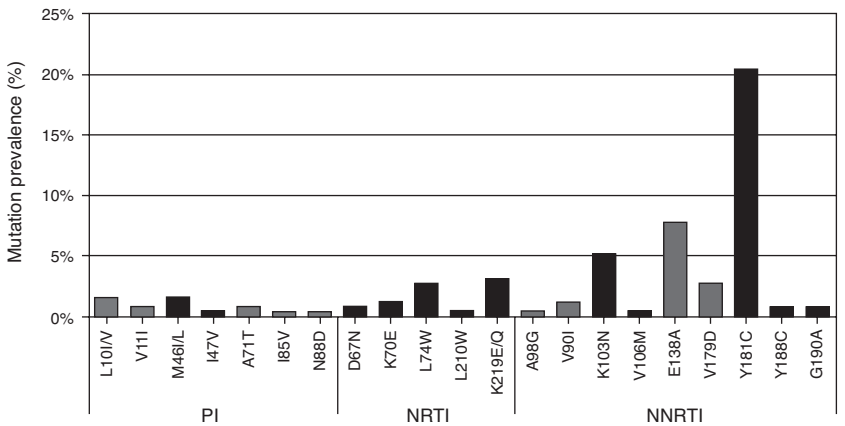

Fig. 1. Prevalence of HIV-1 drug resistance mutations detected by population sequencing among 255 single-dose nevirapine-exposed children initiating antiretroviral therapy. All major protease inhibitor, nucleoside reverse transcriptase inhibitor (NRTI), and nonnucleoside reverse transcriptase inhibitor (NNRTI) mutations are shown (black bars). Minor mutations are shown in gray. Mutations are categorized as per the December 2009 IAS mutation list [24].

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at position 35 in protease. All samples clustered with HIV-1 subtype C sequences except for one which was a subtype A.

Detection of Y181C and K103N minority populations by allele-specific PCR

The Y181C mutation was detected by AS-PCR in all 52 samples that were positive by consensus sequencing as well as an additional 10% (25/250) that were wild-type by sequencing. Two samples had the polymorphisms Y181F/S in the primer binding regions, which interfered with the AS-PCR assay giving false-positive results, and were excluded from analysis. A further three samples were all poorly amplified by AS-PCR, despite no apparent sequence differences in the primer binding regions, so were considered indeterminate and therefore also excluded. The median AS-PCR quantitative value (estimated % of viral population) for the 52 genotype-positive samples was 74.9% (IQR 21.9–97.1), whereas for the 25 AS-PCR only-positive samples, it was 2.8% (IQR 1.0–8.9; Fig. 2).

The K103N mutation was detected in an additional 8% (21/255) of samples by AS-PCR. All of the samples positive for K103N by population sequencing were similarly positive by AS-PCR, with a median quantitative value of 87.8% (IQR 54.3–99.5), whereas for the samples only positive on AS-PCR, it was 1.1% (IQR 0.6–5.0; Fig. 2). The polymorphism K103R was detected by population sequencing in four samples and did not appear to affect the assay in that all four samples were negative by AS-PCR. An additional sample with an unusual K103T polymorphism had a quantitative AS-PCR value of 0.9 for K103N. Similar to K103R, this polymorphism falls out of the primer binding region and, thus, the low positive AS-PCR value was assumed to reflect the presence of K103N.

No samples had both K103N and Y181C detected by population sequence, and only 12 (4.7%) had both mutations detected by either method. Specifically eight samples were positive for Y181C by population sequence and K103N by AS-PCR, two samples were positive for K103N by population sequence and Y181C by AS-PCR, and two samples were positive for both mutations by AS-PCR. In all cases in which both mutations were detected, the infants were less than 6 months of age. One sample had both K103N and Y188C by population genotype, otherwise multiple major NNRTI mutations were not found.

Interestingly, the ratio of all Y181C-containing samples detected only by population sequencing relative to those detected by either method (52/77, 68%) was significantly higher than the ratio of all K103N-containing samples detected only by population sequencing [(13/34, 38.2%), $P = 0.006$]. This indicates that K103N occurred mostly as a minority species and hence the K103N AS-PCR was particularly useful in revealing additional samples harboring this mutation.

Lower prevalence of nonnucleoside reverse transcriptase inhibitor mutations in older children

To assess the presence of NNRTI mutations over time since exposure to sdNVP, the 255 children were categorized into four age groups. Age in children corresponds with length of time since exposure in this case. Analysis of population genotypes showed a clear age trend with 45.7% of infants 0–6 months having major NNRTI mutations, compared to 23.7% at 6–12 months, 20% at 12–18 months, and 0% at 18–24 months ($P < 0.0001$; Table 1) indicating that these mutations fade over time. The Y181C mutation was most common at all time-points and only Y181C and K103N persisted until 18 months. After 18 months, all samples were wild-type by population sequencing. Other major NNRTI mutations V106M, Y188C, and G190A were found in a small proportion of infants under 12 months, but were absent in older children. Polymorphisms classified as minor mutations were generally present at low frequencies and did not vary by age.

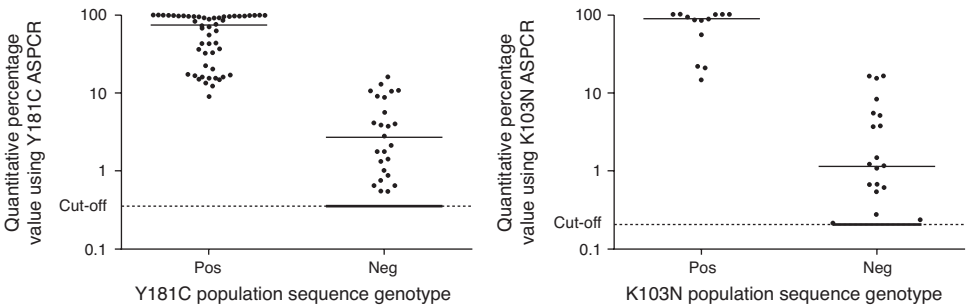

Fig. 2. Comparison of Y181C and K103N allele-specific PCR quantitative value relative to population genotype. The actual quantitative values of the percentage of the viral population found to be Y181C (left panel) and K103N (right panel) by allele-specific PCR (AS-PCR) stratified by genotype result are shown. Solid horizontal lines indicate the median quantitative value of AS-PCR-positive samples in each group. A total of 173 and 221 samples were negative for Y181C and K103N by population sequencing and AS-PCR, respectively.

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Table 1. Nonnucleoside reverse transcriptase inhibitor mutations detected by population sequencing and allele-specific PCR among 255 single-dose nevirapine-exposed children initiating antiretroviral therapy.

	0–6 months	6–12 months	12–18 months	18–24 months	Total	*P* value[a]
Mutation detection						
Total *N* (%) with detectable major NNRTI mutations						
By population sequencing	37 (45.7%)	23 (23.7%)	9 (20.0%)	0	69 (27.1%)	<0.0001
By AS-PCR only	13 (16.0%)	14 (14.4%)	1 (2.2%)	5 (15.6%)	34 (12.9%)	0.13
N (%) with detectable Y181C						
By population sequencing	25 (30.9%)	20 (20.6%)	7 (15.6%)	0	52 (20.4%)	0.0025
By AS-PCR only[b]	10 (12.5%)	11 (11.8%)	1 (2.2%)	3 (9.4%)	25 (10.0%)	0.27
N (%) with detectable K103N						
By population sequencing	10 (12.3%)	1 (1.0%)	2 (4.4%)	0	13 (5.1%)	0.0031
By AS-PCR only	16 (19.8%)	3 (3.1%)	0	2 (6.3%)	21 (8.2%)	<0.0001
N (%) with other major NNRTI mutations						
V106IM	0	1 (1.0%)	0	0	1 (0.4%)	0.65
Y188C	1 (1.2%)	1 (1.0%)	0	0	2 (0.8%)	0.83
G190A	2 (2.5%)	0	0	0	2 (0.8%)	0.23
N (%) with minor NNRTI mutations						
V90I	2 (2.5%)	0	1 (2.2%)	0	3 (1.2%)	0.37
A98G	0	1 (1.0%)	0	0	1 (0.4%)	0.65
E138A	8 (9.9%)	5 (5.2%)	6 (13.3%)	1 (3.1%)	20 (7.8%)	0.23
V179D	1 (1.2%)	4 (5.1%)	2 (4.4%)	0	7 (2.7%)	0.43

AS-PCR, allele-specific PCR; NNRTI, nonnucleoside reverse transcriptase inhibitor.
[a]*P* values determined by comparing percentage of positive samples in each age category (χ^2 test for trend).
[b]One child aged 0–6 months and four children aged 6–12 months were excluded from analyses due to indeterminate Y181C AS-PCR results.

The prevalence of mutations detected only by AS-PCR is shown in Table 1. Detection of Y181C showed no consistent age trend, whereas K103N detected only by AS-PCR had a significantly lower prevalence after the first 6 months of age, suggesting that minority K103N variants do not persist as long as Y181C in infants. Of the five samples that were positive for AS-PCR 18–24 months postexposure to sdNVP but wild-type by sequencing, three were Y181C-positive and two were K103N-positive.

When population sequencing-derived and AS-PCR-derived data were combined, 61.9% of all infants 0–6 months of age harbored NNRTI resistance mutations prior to starting ART therapy (Table 1 and Fig. 3). The frequency of resistance mutations declined significantly over time with 38.6% of 6–12-month-old and 22.2% of 12–18-month-old children showing evidence of NNRTI resistance. Minority variants were still present in 15.6% of children 18–24 months after sdNVP.

Associations between resistance and clinical characteristics

A trend toward higher pretreatment HIV-1 RNA quantities and greater likelihood of detecting NNRTI mutations was found ($P = 0.03$, Table 2). This association was partially explained by age. When stratified into age categories, there was no longer a consistent trend toward increasing resistance with higher pretreatment viral load. Associations between increasing resistance and younger age were observed consistently across all viral load strata. There was no association between resistance and CD4 percentage, child sex, any breastfeeding, and clinical stage.

In 71 children switched from the initial protease inhibitor-based regimen to NNRTI-based therapy and who had pretreatment samples available, nine of 25 (36.0%) with Y181C or K103N detected by AS-PCR had confirmed viremia more than 1000 copies/ml within 52 weeks of regimen change compared to five of 46 (10.9%) of those with no detectable mutations ($P = 0.01$). If those with NNRTI mutations detected by population sequencing were excluded, there was no significant difference in the risk of failure between those with

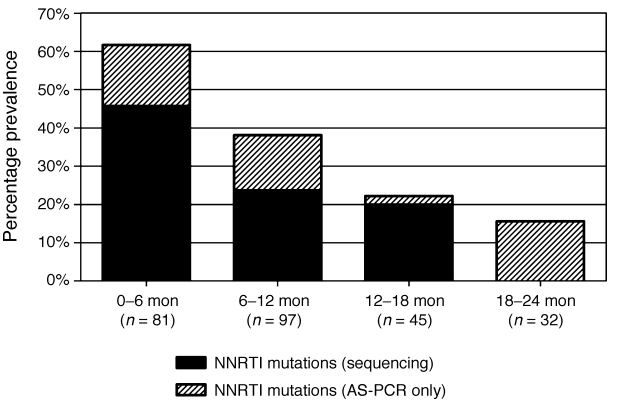

Fig. 3. Overall prevalence of nonnucleoside reverse transcriptase inhibitor mutations among 255 single-dose nevirapine-exposed children initiating antiretroviral therapy. Data show the prevalence of nonnucleoside reverse transcriptase inhibitor (NNRTI) mutations by genotyping (black bars) and the additional prevalence when including samples that were only identified using the Y181C or the K103N allele-specific PCR (AS-PCR; gray bars). Samples classified as indeterminate by Y181C AS-PCR were excluded.

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Table 2. Associations between pretreatment nonnucleoside reverse transcriptase inhibitor resistance mutations detected by population sequencing and allele-specific PCR and clinical characteristics.

Clinical characteristics	Y181C by population genotype	K103N by population genotype	Y181C by AS-PCR only[a]	K103N by AS-PCR only	Other major NNRTI mutations	No NNRTI mutations detected	*P* value[b]
Age of child							
0–12 months	45/178 (25.3%)	11/178 (6.2%)	18/173 (10.4%)	9/178 (5.1%)	4/178 (2.2%)	86/178 (48.3%)	
12–24 months	7/77 (9.1%)	2/77 (2.6%)	4/77 (5.2%)	2/77 (2.6%)	0	62/77 (80.5%)	<0.0001
Sex							
Male	26/127 (20.5%)	3/127 (2.4%)	10/124 (8.1%)	5/127 (3.9%)	2/127 (1.6%)	78/127 (61.4%)	
Female	26/128 (20.3%)	10/128 (7.8%)	12/126 (9.5%)	6/128 (4.7%)	2/128 (1.6%)	70/128 (54.7%)	0.29
Pretreatment CD4%							
0–20	33/141 (23.4%)	6/141 (4.3%)	14/138 (10.1%)	5/141 (3.5%)	3/141 (2.1%)	77/141 (54.6%)	
>=20	19/114 (16.7%)	7/114 (6.1%)	8/112 (7.1%)	6/114 (5.3%)	1/114 (0.9%)	71/114 (62.3%)	0.28
Pre-Rx viral load[c]							
<100 000 copies/ml	0	1/17 (5.9%)	1/16 (6.3%)	0	0	14/17 (82.4%)	
100 000–750 000	10/60 (16.7%)	3/60 (5.0%)	4/59 (6.8%)	5/60 (8.3%)	1/60 (1.7%)	36/60 (60.0%)	0.0314
≥750 000 copies/ml	38/160 (23.8%)	9/160 (5.6%)	16/157 (10.2%)	4/160 (2.5%)	2/160 (1.3%)	88/160 (55.0%)	
Clinical stage[d]							
I	5/39 (12.8%)	3/39 (7.7%)	2/37 (5.4%)	3/39 (7.7%)	0	24/39 (61.5%)	
II	1/11 (9.1%)	0	2/11 (18.2%)	1/11 (9.1%)	0	7/11 (63.6%)	0.25
III	27/126 (21.4%)	5/126 (4.0%)	12/123 (9.8%)	3/126 (2.4%)	2/126 (1.6%)	74/126 (58.7%)	
IV	18/76 (23.7%)	5/76 (6.6%)	6/76 (7.9%)	4/76 (5.3%)	2/76 (2.6%)	41/76 (53.9%)	

[a]Five Y181C allele-specific PCR (AS-PCR) indeterminate samples were excluded from analysis.
[b]*P* values determined by comparing all participants with nonnucleoside reverse transcriptase inhibitor (NNRTI) mutations to those without (χ^2 tests).
[c]Eighteen samples had no corresponding viral load data.
[d]Three samples had no corresponding clinical staging data.

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mutations detectable only by AS-PCR [one of five (20%) children failed] and those with no mutations [five of 46 (10.9%) failed, $P = 0.55$]. Detection of Y181C or K103N by AS-PCR at an estimated frequency more than 25% of the viral population was associated with a significantly increased risk of failure [eight of 18 (44.4%) failed], whereas Y181C or K103N detected in less than 25% of the viral population was not [one of seven (14.3%) failed] relative to those with no mutations ($P = 0.003$ and $P = 0.79$, respectively).

Discussion

Supported by the results of a recent trial [25], WHO pediatric treatment guidelines recommend use of boosted protease inhibitor-based therapy to all children under 2 years of age if NVP-exposed [26]. Consistent with these guidelines, all children in our cohort initiated protease inhibitor therapy. As expected, there was no association between NNRTI mutations at treatment initiation and virological failure during the primary regimen [27,28]. However, after switching to NVP-based therapy, those with pretreatment NNRTI mutations detected using population sequencing were more likely to fail [21]. Here we report that low-level NNRTI mutations missed by population sequencing but detectable by AS-PCR did not influence response to the re-use of NVP. Mutations detectable by AS-PCR but present in less than 25% of the viral population were not associated with failure. These findings are consistent with our prior results using ultra-deep pyrosequencing that also observed that pretreatment NNRTI mutations when present below 25% of the viral population did not affect response to NVP-based therapy when preceded by suppression on the boosted protease inhibitor-based regimen [20]. We hypothesize that initial suppression first on the LPV/r-based regimen may raise the threshold of the frequency of resistance mutations required to result in clinically significant differences in virologic response to therapy.

The samples were analyzed using conventional population sequencing as well as allele-specific real-time PCR assays. Although population sequencing remains the gold standard and provides a comprehensive description of all amino acids in the regions analyzed, it is comparatively insensitive, detecting resistant species in heterogeneous populations only when they are above 20% [29,30]. Our data confirm that AS-PCR, with its detection levels of approximately 1% of minority species, provides a significantly more accurate representation of the extent and persistence of mutant species, though confined to one mutation at a time. In this population, analysis of the Y181C and K103N mutations by AS-PCR was sufficient, as other NNRTI mutations were rare. The high sensitivity and relative simplicity of the AS-PCR assay makes this methodology an attractive one for investigating drug resistance in samples in which the mutational patterns can be anticipated. When considering whether it is safe to switch NVP-exposed children initially suppressed on a LPV/r-based regimen, standard genotyping already approved for clinical use would be adequate. However, as AS-PCR is less expensive, these assays could be developed for clinical use and in this application, a mutation frequency of more than 20% would suffice.

Consistent with other pediatric studies, the Y181C mutation was most frequently detected [2,9,11,14]. This is in contrast to studies in adult women in whom K103N predominates [2–6]. It is unclear why the virus transmitted to children develops different mutations to the ones present in their mothers, but possible reasons include higher viral loads in infants and greater drug exposure due to the fact that children receive in-utero exposure in addition to oral dosing after birth. In children, Y181C detected either by standard methods [25] or ultra-sensitive methods [16,17] has been associated with attenuated virologic response to primary NVP-based therapy. Intriguing new results in adults suggest that low frequency Y181C does not compromise response to efavirenz-based therapy [31]. Unfortunately, for young children, efavirenz cannot be used due to difficulty establishing suitable therapeutic doses for children under 3 years or less than 10 kg. Thus, this hypothesis cannot be tested in this young age group.

The detection of NRTI and protease inhibitor mutations in some sd-NVP-exposed children prior to starting therapy was unexpected. To the best extent of our information, these infants and mothers had not been exposed to antiretroviral drugs either during pregnancy or breastfeeding, other than sdNVP. Studies in South Africa have indicated that levels of transmitted resistance remain low [22], suggesting that in some cases these may be naturally occurring mutations. Unusual profiles of mutations, unrelated to the drugs used for prophylaxis, have also been noted among infants in the United States [32].

Analysis of the samples using both population sequencing and the more sensitive AS-PCR allowed us to detect interesting differences between the dynamics of Y181C and K103N mutations in children. With Y181C, more than two-thirds of mutations were detected by population sequencing, whereas with K103N, the reverse pattern was observed, with more than 60% of K103N mutations detected only with the more sensitive AS-PCR. Although the prevalence of both Y181C and K103N mutations declined with age, the steepness of the decline was more marked with K103N. Y181C appears to be a more persisting mutation in children. Longer periods after exposure may be necessary in children before NNRTI mutations decline below the detection threshold.

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It was initially hypothesized that the clinical consequences of sdNVP in compromising virologic response to primary NNRTI therapy in women may be completely attenuated within 6 months of exposure [33]. Subsequent studies suggested that this wash-out period is more likely to be 12–18 months [34–36]. Studies in infants similarly support poor virologic responses to NNRTI-based therapy in sdNVP-exposed infants, especially when initiating therapy before the age of 12 months [16,33], although this is not consistently observed [37]. Recent evidence indicates that a delay between exposure and treatment initiation should be avoided in infants. A landmark study demonstrated that infants fared better if treatment was initiated soon after diagnosis rather than waiting until clinical indicators were met [38]. Thus, clinical programs should initiate therapy for infants as early as possible, which is also the time when the detection of resistance mutations is at its highest and most compromising.

Pretreatment drug resistance screening is recommended in many resource-rich environments. It would be helpful to identify which children could be safely initiated on NVP-based therapy despite their history of exposure. The high costs and limited laboratory capacity, as well as the challenges of returning results to clinical sites rapidly so that treatment can be started almost immediately, preclude routine screening in any public service in sub-Saharan Africa to our knowledge. Our data also indicate that in children less than 6 months of age, over 60% have detectable resistance mutations. The costs of screening would need to be balanced against the money saved by initiating the less expensive regimen in the remaining 40%. Newer pMTCT regimens and more widespread use of maternal therapy may reduce the prevalence of NNRTI mutations among infected children making for a more favorable cost–benefit ratio of pretreatment resistance screening.

A limitation of this study is its cross-sectional nature. We do not have data on what the prevalence of mutations was soon after sdNVP exposure to confirm in a longitudinal design that mutations faded over time. Nevertheless, we did not detect associations between markers of increased severity of disease and resistance once age was taken into account. Moreover, our sample is informative for its generalizability to clinical practice as it included a large cohort who had been exposed to sdNVP as used as part of routine public health programs. Thus, it provides a useful estimate for policy purposes of the extent to which it is necessary to avoid primary NNRTI-based therapy in infants presenting at different ages who report a history of sdNVP exposure. These estimates may be useful for programs that do not have sufficient resources to provide boosted protease inhibitor-based regimens to all exposed children who could ration use of this regimen to the youngest children only. Our estimates may also be useful for cost-effectiveness analyses of drug resistance testing in low-resource settings.

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