Towards virological monitoring of HIV-1 drug resistance in resource-limited settings
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Accumulation of drug resistance and loss of therapeutic options precede commonly used criteria for treatment failure in HIV-1 subtype C infected patients

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Abstract

Virological monitoring is essential for early identification of antiretroviral treatment (ART) failure. Here, the accumulation of resistance in African HIV-1 subtype C infected patients with ongoing viral replication during ART was determined and consequences for second-line therapy were estimated. 836 patients initiated NNRTI-based ART and received biannual HIV-RNA monitoring. When first-line ART was continued despite virological failure (HIV-RNA >1000 copies/mL), genotypic resistance analysis was performed at baseline, first failure (t1), and 6 or 12 months later (t2). Major resistance mutations (IAS), Stanford genotypic sensitivity scores (GSSs) and proportions of patients meeting WHO-defined failure criteria were compared between time-points. Most patients (642/836, 77%) reached viral suppression. 145/642 patients (23%) experienced subsequent failure, after a median of 18 months. Counselling resulted in virological re-suppression in 27% (39/145); 40% (58/145) continued first-line ART despite virological failure. 26 patients were included for genotypic analysis. The mean number of major drug-resistance mutations per person increased from 2.8 (t1) to 4.3 (t2). Initially, NNRTI-associated mutations (n=47) predominated; only 25 NRTI-associated mutations (mainly M184V) were detected. During prolonged viremia, NRTI-resistance increased (n=44, +76%), in particular TAMs (from 4 to 14) and K65R (from 3 to 6). Consequently, GSSs declined from baseline to t1 and t2: from 3.8 to 1.0 to 0.7 (NNRTIs) and from 6.8 to 5.1 to 4.0 (NRTIs). Despite broad resistance, immunological failure was limited at t2. Rapid accumulation of drug resistance occurred when ART was continued despite virological failure. Treatment options were lost, even when WHO-defined failure criteria were not met. This study calls for wider access to virological monitoring.
Introduction

The number of people receiving antiretroviral therapy (ART) has increased considerably over the last decade. Worldwide, nearly four million people were reported to receive treatment by the end of 2008. The majority of these patients live in Sub-Saharan Africa, the region hit hardest by the HIV-pandemic\(^\text{(1)}\). The extensive roll-out of ART has dramatically improved life expectancy of HIV-infected individuals residing there\(^\text{(2, 3)}\). Moreover, despite high early mortality rates, short-term virological outcomes of Africa ART programmes are good. Overall, the majority of patients achieve virological suppression within a year of commencing treatment. In spite of these impressive results, not all patients show continued virological control; within a few years after initial suppression, around 15% of patients experience virological failure\(^\text{(4)}\). In these patients, drug resistance mutations can be selected due to ongoing viral replication during antiretroviral (ARV) drug pressure.

To limit the occurrence of drug-resistance, western treatment-guidelines recommend frequent HIV viral load testing (every 2-3 months) for all patients initiating ART\(^\text{(5)}\). Regular monitoring enables resistance testing and switching treatment as soon as HIV-RNA levels rise. Consequently, further selection of drug-resistance and loss of therapeutic options may be prevented. Unfortunately, such frequent monitoring is generally not feasible in low income countries (LICs). The World Health Organization’s (WHO) guidelines therefore recommend a lower monitoring frequency in LICs (biannually). When virological diagnostics are not available, it is advised to use immunological or clinical parameters to guide treatment decisions (6, 7). Unfortunately, the correlation between these parameters and virological treatment outcomes is marginal\(^\text{(8-10)}\).

Due to irregularities in virological monitoring and the poor predictive values of immunological and clinical decline for virological failure, the detection of patients experiencing treatment failure is delayed. Moreover, the limited
number of available treatment options in LICs renders caregivers reluctant to switch ART, even if a viral load increase is identified. Previous studies in LICs have shown the number of drug-resistance mutations to be associated with the duration of ART exposure and with persistent viremia\(^{11-13}\). The rate at which the prolonged use of failing ART during viral replication affects viral drug-resistance profiles within individual patients is however not described.

In this study, we evaluate accumulation of drug-resistance during first-line ART in a cohort of South African HIV-1 subtype C infected patients. Subsequently, we placed our data in the context of WHO-defined failure criteria.

**Methods**

*Site and Patients*

Patients receiving ART at the Ndlovu medical centre (NMC, www.ndlovucaregroup.com) were included. Details on NMC and its ART programme have been described elsewhere\(^ {14}\). In short, NMC is located in the poor, rural Limpopo province of South Africa. A fully funded ART programme was initiated in 2003. For adult patients, a WHO clinical stage IV and a CD4+ T-cell count below 200 cells/mm\(^3\) were used as criteria for treatment eligibility. For children, apart from clinical criteria, a percentage of CD4+ T-cells (CD4\%) <15\% was used to initiate ART. First-line ART consisted of efavirenz or nevirapine plus stavudine or zidovudine and lamivudine. Patients receive individual counselling at each clinic visit; both prior to and during the use of ARVs.

Second-line therapy is available for patients experiencing virological failure and considered to be adherent to treatment. The exact timing of a treatment switch is based on WHO-defined criteria, or performed earlier at the doctors’ descretion. Second-line therapy is comprised of the boosted protease inhibitor (PI) lopinavir/ritonavir and a nucleoside reverse transcriptase inhibitor (NRTI) backbone.
**Study population**

For this study, all ARV-naïve patients who started first-line ART at least two years prior to data-collection were eligible. Patients who were known to have received single dose nevirapine as prevention of mother to child transmission prior to starting ART were excluded. Virological failure (HIV-RNA >1000 copies/mL) after initial virological suppression, was subsequently used as patient-selection criterion. When first-line ART was continued despite ongoing viral replication, patients were considered to risk accumulation of drug-resistance mutations. Longitudinal genotypic analysis was performed when plasma samples were available at first detection of virological failure and 6 or 12 months later.

**Data collection**

Regular on-site laboratory monitoring was available for all patients initiating ART. Absolute and relative CD4+ T-cell counts (FACSCount system, Becton Dickinson Biosciences, San Jose, CA), as well as plasma HIV viral load levels (system 340 bDNA analyzer, Bayer AG, Leverkusen, Germany, limit of detection 50 copies/mL) were determined before treatment, three times during the first, and biannually during subsequent years of treatment. Standard methods for blood sample processing were used. From each blood draw, 1 mL of plasma was stored at -80 °C for future analysis.

Genotypic resistance analysis was performed on plasma HIV-1 RNA by population based sequence analysis for the protease and RT genes in a WHO-accredited HIV drug-resistance laboratory in the Netherlands, using an in-house developed protocol\(^{(15)}\). HIV-subtypes were determined for the generated consensus sequences using the Los Alamos HIV-1 sub-typing tool\(^{(16)}\).
Definitions

Virological suppression was defined as an HIV-RNA viral load below the level of detection (<50 copies/mL). Virological failure was defined as an HIV-RNA viral load increase to >1000 copies/mL after initial suppression. For patients who continued first-line ART, ongoing viremia was defined as a second viral load measurement >400 copies/mL, at least 6 months after first detection of virological failure.

Virological failure according to WHO criteria was defined as an HIV-RNA viral load increase to >5000 copies/mL after at least three months of ART. Immunological failure according to WHO criteria was defined as a fall of CD4+ T-cell count to pre-therapy level, a 50% drop from the on-treatment peak value, or persistent CD4+ T-cell levels <100 cells/mm$^3$. For children, a sustained decline of five percentage points in CD4% at any age, or decline to the pre-therapy CD4+ T-cell count when older than 5 years of age, were used to define immunological failure.

A baseline blood sample refers to a blood sample that was collected after an HIV infection was established and prior to the initiation of first-line ART. Time point one (t1) was when virologic failure was first detected. Time point two (t2) was after 6 or 12 months of ongoing viremia.

Observed genetic profiles were analyzed according to the IAS 2009 update of drug-resistance mutations. Thymidine analogue mutations (TAMs) were M41L, D67N, K70R, L210W, T215Y/F and K219Q/E. Tenofovir-associated mutations were K65R and K70E. All TAMs and K65R were considered multi-NRTI resistance mutations.

Statistical analysis

Kaplan-Meier survival analysis was used to estimate time from treatment start to viral suppression and from viral suppression to subsequent failure. Proportions of patients with various specific drug-resistance profiles were
calculated at baseline, t1 and t2.

Phylogenetic analysis was performed using a neighbour joining tree with 1000 boots. In order to estimate viral susceptibility to NRTIs and NNRTIs, genotypic sensitivity scores (GSSs)\(^{(19,20)}\) were calculated at baseline and at both failure time points. Drugs that were deemed fully active or were labeled with potential resistance according to the Stanford HIV drug-resistance database\(^{(21)}\) (accessed 2010) were scored as “1”; drugs for which low or intermediate resistance was present as “0.5” and in case of complete resistance as “0”.

Several determinants (being a child; NRTI backbone; NNRTI used; duration between t1 and t2, and WHO-defined virological or immunological failure) were analysed as possible predictors for the selection of multi-NRTI resistance mutations. Proportions of patients harbouring multi-NRTI resistant virus were compared between groups using the chi-square (\(\chi^2\)) test. These proportions were subsequently used to calculate positive and negative predictive values (PPVs and NPVs) of WHO failure criteria for the selection of multi-NRTI resistance mutations. Statistical analyses were performed using SPSS 15.0.

**Results**

*Cohort description*

A total of 836 patients were included, 101 (12%) of whom were children. Median duration since treatment initiation was approximately three years. Most patients were female and suffered from severe immuno-suppression when starting ART, as is shown by the low baseline CD4+ T-cell counts in adults (median 68 cells/mm\(^3\)) and CD4% in children (median 11%). While efavirenz was the most commonly used NNRTI in adults, children frequently received nevirapine. All children received a zidovudine/lamivudine NRTI-backbone, whereas 79% of adult patients received a stavudine-containing regimen. *Table 1* summarizes baseline characteristics.
Table 1. Patient characteristics at time of initiating ART

<table>
<thead>
<tr>
<th></th>
<th>All adults n = 735</th>
<th>All children n = 101</th>
<th>Continued viral failure; resistance test done n = 26</th>
<th>Continued viral failure; no resistance test done n = 32</th>
</tr>
</thead>
<tbody>
<tr>
<td>Median age, years (IQR)</td>
<td>34 (29-41)</td>
<td>4.8 (2.9-7.0)</td>
<td>30 (24-34)</td>
<td>7.0 (5.5-7.3)</td>
</tr>
<tr>
<td>Female gender, n (%)</td>
<td>526 (72)</td>
<td>57 (56)</td>
<td>15 (79)</td>
<td>4 (57)</td>
</tr>
<tr>
<td>Median weight, kg (IQR)</td>
<td>52 (45-60)</td>
<td>14 (11-17)</td>
<td>51 (44-66)</td>
<td>18 (13-18)</td>
</tr>
<tr>
<td>Median CD4(^+) T-cell count, cells/mm(^3), (IQR)</td>
<td>68 (20-140)</td>
<td>244 (130-666)</td>
<td>68 (25-129)</td>
<td>198 (56-244)</td>
</tr>
<tr>
<td>Median CD4%, % (IQR)</td>
<td>NA</td>
<td>11.0 (4.7-5.5)</td>
<td>NA</td>
<td>6.2 (2.0-11)</td>
</tr>
<tr>
<td>Median HIV RNA log(_{10}) copies/ml (IQR)</td>
<td>5.0 (4.5-5.4)</td>
<td>4.9 (4.6-5.5)</td>
<td>4.9 (4.5-5.3)</td>
<td>5.2 (4.9-5.4)</td>
</tr>
<tr>
<td>NNRTI administered</td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Efavirenz, n (%)</td>
<td>427 (58)</td>
<td>26 (26)</td>
<td>8 (42)</td>
<td>4 (57)</td>
</tr>
<tr>
<td>Nevirapine, n (%)</td>
<td>303 (41)</td>
<td>74 (73)</td>
<td>11 (58)</td>
<td>3 (43)</td>
</tr>
<tr>
<td>Unknown, n (%)</td>
<td>5 (1)</td>
<td>1 (1)</td>
<td>0</td>
<td>0</td>
</tr>
<tr>
<td>NRTI administered</td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Zidovudine (+ lamivudine), n (%)</td>
<td>151 (21)</td>
<td>101 (100)</td>
<td>7 (37)</td>
<td>7 (100)</td>
</tr>
<tr>
<td>Stavudine (+ lamivudine), n (%)</td>
<td>577 (79)</td>
<td>0</td>
<td>12 (63)</td>
<td>0</td>
</tr>
<tr>
<td>Unknown, n (%)</td>
<td>7 (1)</td>
<td>0</td>
<td>0</td>
<td>0</td>
</tr>
<tr>
<td>Median duration of follow-up(^a), months (IQR)</td>
<td>35 (29-45)</td>
<td>31 (22-48)</td>
<td>33 (29-51)</td>
<td>27 (19-39)</td>
</tr>
</tbody>
</table>

\(^a\)Time between antiretroviral therapy (ART) initiation and moment of data collection. CD4\%, percentage of CD4-positive T-cells; IQR: inter-quartile range; NA, not available or not applicable; n, number of patients; NNRTI, non-nucleoside reverse transcriptase inhibitor; NRTI, nucleoside reverse transcriptase inhibitor.
Seventy seven percent (642/836) of all patients achieved virological suppression on first-line ART, a median of 3 months after treatment start. The majority of patients not reaching virological suppression died soon after starting ART (116/194, 60%), probably reflecting highly advanced HIV disease presentation. The six patients who remained in care, but did not reach virological suppression on first line ART were considered to be non-adherent to treatment and also excluded from analysis. After initial virological suppression, 145/642 patients (23%) experienced a viral rebound to over 1000 copies/mL. An immediate treatment switch to second-line ART was made in 33% (48/145) of these patients. Additional counselling without switching treatment resulted in virological re-suppression in 27% (39/145) of individuals who were initially suspected of virological failure. Ongoing viremia during first-line ART was documented in the remaining 58 patients (40%).

Plasma samples both at first detection of virological failure and at a later stage during first-line ART were available for 40 of 58 patients with ongoing viremia. Twenty-six patients with either 6 (n=18) or 12 (n=8) months between blood draws were analyzed for possible accumulation of drug-resistance mutations. For the remaining 14 patients, the time span between t1 and t2 was longer than 12 months. These patients were therefore excluded from genotypic analysis (Figure 1). Baseline characteristics of patients who continued viral failure who were included in genotypic analysis were similar to the baseline characteristics of patients with continued viral failure who were not included for resistance testing (Table 1) All individuals harboured HIV-1 subtype C. Figure 1 shows a flow chart of patient selection and reasons for exclusion.
Figure 1. Patient selection flow chart

Drug-resistance profiles
Median viral load at t1 was 6931 copies/mL (inter-quartile range [IQR] 3427-40039 copies/mL) and 12328 copies/mL (IQR 3027-33551 copies/mL) at t2. As expected, phylogenetic analysis revealed strong clustering between plasma samples from individual patients.

At first detection of virological failure (t1), 92% (24/26) of viral isolates expressed major drug-resistance mutations in reverse transcriptase. All patients harboured drug-resistant HIV at t2 (Table 2). Initially, NNRTI-associated mutations predominated (47/72, 65%); only 25 NRTI-associated mutations were detected at t1, mainly M184V (15 times).
The total number of mutations increased substantially from t1 to t2 (72 to 111, +54%); the mean number of major drug-resistance mutations per person increased from 2.8 to 4.3. Prolonged virological failure resulted in a predominant increase of NRTI-associated mutations (from 25 to 47 mutations, +88%). NNRTI-associated mutations, which were already highly prevalent at t1, only showed a 36% increase (Figure 2A).

Figure 2 A. Drug resistance according to drug classes

The mutations M184V and K103N were the most frequently observed mutations at both time points, but the proportion of patients harbouring these mutations were only slightly higher at t2 (M184V 73% [19/26]; K103N 65% [17/26]), compared to t1 (M184V 58% [15/25]; K103N 46% [12/26]). In contrast, a steep increase in TAMs (from 4 to 14 mutations, +250%) was observed. The K65R frequency also doubled between t1 and t2 (from 3 to 6, Figure 2B).

TAMs were predominantly selected in zidovudine-receiving patients; 57% (8/14) ultimately harboured such mutations, compared to 8% (1/12) in stavudine-receiving individuals (p<0.01). On the other hand, K65R was only detected in stavudine-receiving patients; half of viral isolates (6/12) ultimately expressed this mutation.
**Table 2. Drug resistance profiles in individual patients**

<table>
<thead>
<tr>
<th>ART Regimen</th>
<th>Baseline Resistance</th>
<th>Drug resistance Profiles at t1(^a) and t2(^b)</th>
<th>TAMs / K65R</th>
</tr>
</thead>
<tbody>
<tr>
<td>1</td>
<td>CBV/NVP(^c)</td>
<td>K103N, <strong>E138Q</strong>, M184V, T215NSY</td>
<td>1 TAM</td>
</tr>
<tr>
<td>2</td>
<td>CBV/NVP(^c)</td>
<td>K103N, M184I, G190A</td>
<td>0</td>
</tr>
<tr>
<td>3</td>
<td>CBV/NVP(^c)</td>
<td>M41L, A98G, K103N, E138A, M184V, T215FS → T215F</td>
<td>2 TAMs</td>
</tr>
<tr>
<td>4</td>
<td>CBV/NVP</td>
<td>V106A, M184V, <strong>F227L</strong></td>
<td>0</td>
</tr>
<tr>
<td>5</td>
<td>CBV/NVP</td>
<td>K103N, V106M(^a), E138A, F227L(^a)</td>
<td>0</td>
</tr>
<tr>
<td>6</td>
<td>CBV/NVP</td>
<td>A62V(^a), K101E(^a), K103N(^a), K103S, V106M(^a), <strong>M184V</strong>, G190A</td>
<td>0</td>
</tr>
<tr>
<td>7</td>
<td>CBV/EFV(^c)</td>
<td>V106M, E138, M184V, T215F, F227L, M230L</td>
<td>1 TAM</td>
</tr>
<tr>
<td>8</td>
<td>CBV/EFV(^c)</td>
<td>K103N, <strong>V108I</strong>, M184V, T215F, M230L</td>
<td>1 TAM</td>
</tr>
<tr>
<td>9</td>
<td>CBV/EFV(^c)</td>
<td><strong>D67N</strong>, K70R, K103N, M184V, T215F, K219Q, P225H</td>
<td>4 TAMS</td>
</tr>
<tr>
<td>10</td>
<td>CBV/EFV(^c)</td>
<td><strong>K101Q</strong>, K103N, M184V, T215Y, M230L</td>
<td>1 TAM</td>
</tr>
<tr>
<td>11</td>
<td>CBV/EFV</td>
<td>K101P, K103N → K103S, M184V</td>
<td>0</td>
</tr>
<tr>
<td>12</td>
<td>CBV/EFV</td>
<td><strong>D67N</strong>, V90I, K103N, V106M(^a), E138A</td>
<td>1 TAM</td>
</tr>
<tr>
<td>13</td>
<td>CBV/EFV</td>
<td><strong>D67N</strong>, K70E, V106M, M184V, G190A</td>
<td>1 TAM</td>
</tr>
<tr>
<td>14</td>
<td>CBV/EFV</td>
<td>K103N, E138G, <strong>M184V</strong></td>
<td>0</td>
</tr>
<tr>
<td>15</td>
<td>D4T/3TC/NVP</td>
<td><strong>K65R</strong>, K70E, A98G, K103N, Y181C</td>
<td><strong>K65R</strong></td>
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<tr>
<td>16</td>
<td>D4T/3TC/NVP</td>
<td>K103N, Y181C, M184V</td>
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<tr>
<td>17</td>
<td>D4T/3TC/NVP</td>
<td><strong>K101E</strong>, V108I, Y181C, M184V</td>
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<tr>
<td>18</td>
<td>D4T/3TC/NVP</td>
<td>V90I</td>
<td><strong>K65R</strong></td>
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<tr>
<td>19</td>
<td>D4T/3TC/NVP</td>
<td>K65R, K101E(^a), <strong>V108I</strong>, Y181C, <strong>M184V</strong></td>
<td><strong>K65R</strong></td>
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<tr>
<td>20</td>
<td>D4T/3TC/NVP</td>
<td><strong>K101E</strong>, K103N(^a), M184V, G190A</td>
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<tr>
<td>21</td>
<td>D4T/3TC/NVP</td>
<td><strong>K103R</strong>, V197D, Y181C(^a), M184V, Y188L</td>
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<tr>
<td>22</td>
<td>D4T/3TC/NVP</td>
<td>A62V(^a), K65R, <strong>T69d</strong>, K103N, V106M(^a), Y181C, G190RS(^a), <strong>K219R</strong></td>
<td><strong>K65R</strong></td>
</tr>
<tr>
<td>No.</td>
<td>ART Regimen</td>
<td>Drug Resistant Mutations</td>
<td>Time Period</td>
</tr>
<tr>
<td>-----</td>
<td>----------------</td>
<td>-----------------------------------</td>
<td>-------------</td>
</tr>
<tr>
<td>23</td>
<td>D4T/3TC/EFV</td>
<td>L74V, V75L*, K103N, V108I, M184V, M230L, <strong>L234I</strong></td>
<td>0</td>
</tr>
<tr>
<td>24</td>
<td>D4T/3TC/EFV</td>
<td>K65R, L100I, K103N, T215I</td>
<td>K65R</td>
</tr>
</tbody>
</table>

*Mutations found at first detection of virological failure (t1) but no longer detectable after 6 or 12 months of ongoing viremia (t2). The mutations in bold are the mutations that were only observed at t2. Mutations depicted in italics are drug resistance mutations that are not listed in IAS list. Arrows indicate a change of mutations at one locus between t1 and t2. Patient was a child. ART, antiretroviral therapy; CBV, combivir; d4T, stavudine; EFV, efavirenz; NA, not available; NVP, nevirapine; TAM, thymidine analogue mutation; 3TC, lamivudine. n, number of observed TAMs per patient.
Baseline plasma samples were available for 19/26 (73%) patients. Most (15/19, 79%) harboured wild-type virus, but in four cases major resistance mutations were detected prior to initiating ART. In two instances, mutations were present at polymorphic sites (V90I, E138A) and may represent natural variation rather than previous ARV exposure. Still, these mutations may play a role in resistance to newer NNRTIs (etravirine and rilpivirine), as minor and major mutations for these drugs are not yet clearly defined. In the remaining two patients ART efficacy was already hampered at baseline, due to the tenofovir-related K70E mutation in one, and the presence of multiple mutations (V106M, M184V and G190A) in the other patient.

**Predictors for multi-NRTI drug-resistance**

None of the analysed determinants were significantly associated with the selection of multi-NRTI resistance mutations. However, at t2, a trend towards a higher prevalence of such mutations amongst children compared to adult patients was observed (86% [6/7] versus 47% [9/19], P=0.08). This trend was less evident at first detection of viremia (29% [2/7] versus 21% [4/19], P=0.69).

*Figure 2 B. Specific drug resistance mutations*
At t1, WHO-defined immunological and virological failure was only present in 28% (7/25 patients with data available) and 58% (15/26) of individuals respectively. After prolonged failure (t2), the proportion of patients with WHO-defined virological failure had increased to 85%, whereas the majority of patients still did not show immunological failure (Figure 3). There was no substantial association between the appearance of multi-NRTI resistance mutations and WHO criteria for treatment failure; all four patients without WHO-defined virological failure did harbour a virus with multi-NRTI resistance mutations, and half (8/16) of the viral isolates in patients without immunological failure expressed such mutations. The NPV of immunological failure criteria for the presence of multi-NRTI resistance mutations was therefore only 50%. PPVs to detect such mutations were 50% and 70% for WHO-defined virological and immunological failure respectively.

**Figure 3.** Drug resistance in relation to WHO failure criteria

**Genotypic sensitivity scores**

The number of treatment options declined during ongoing viremia. At baseline most viruses (17/19, 89%) were fully susceptible to both NRTIs (mean GSS=6.8, 97% of maximum score) and NNRTIs (GSS=3.8, 95% of maximum score). At t1, the mean GSS for NNRTIs had already declined to 1.0 (25% of maximum score), whereas the mean NRTI-GSS was still 5.1 (73% of maximum score). Between t1 and t2, the mean GSSs decreased
even further to 0.7 (18% of maximum score) for NNRTIs and 4.0 (57% of maximum score) for NRTIs. Figure 4 shows the predicted viral susceptibility per individual drug. NRTI susceptibility clearly declines between t1 and t2, whereas NNRTI resistance is already highly prevalent at first detection of virological failure. Due to the high number of NNRTI resistance mutations, the efficacy of newer NNRTIs such as etravirine, is estimated to be hampered in most patients (22/26; 86%); 10 viral isolates were regarded as having low-level resistance to these drugs, 11 as having intermediate resistance and one patient harboured a virus that was deemed NNRTI resistant.

Figure 4. Predicted viral drug susceptibility. ABC, abacavir; AZT, zidovudine; EFV, efavirenz; ETR, etravirine; FTC, emtricitabine; d4T, stavudine; ddI, didanosine; NVP, nevirapine; TDF, tenofovir; t1, first detection of virological failure; t2, after 6 or 12 months of ongoing viremia; 3TC, lamivudine.

Discussion
This study describes good virological outcomes in the majority of HIV-1 subtype C infected patients receiving first-line ART in a rural, African setting. These results are comparable to those observed in other African cohorts\(^4\). However, in patients experiencing virological failure, a rapid accumulation
of major drug-resistance mutations was observed when first-line ART was continued despite ongoing viremia. Accumulation of resistance was mainly caused by an increased selection of NRTI-associated mutations. The high number of multiple-NRTI associated mutations (TAMs and K65R) in a background of existing NNRTI mutations clearly limits future treatment options in patients with ongoing viremia. The correlation between immunological decline and virological failure is reported to be limited(8-10). Our results now demonstrate that even after prolonged viral replication, and despite clear accumulation of drug-resistance, immunological failure is still only apparent in a minority of patients. To our knowledge, this is the first study to describe longitudinal genotypic analysis data in individual HIV-1 subtype C infected patients during continued failing first-line ART.

Subtype C is the most prevalent HIV-1 subtype in Sub-Saharan Africa. The vast majority of African patients receiving ART are treated with NNRTI-based, first-line regimens(4). The data presented in this paper may therefore have widespread implications. However, one should not immediately conclude that all patients receiving ART in Africa need to be switched to a second-line regimen at first detection of viremia. Although an early treatment switch would reduce resistance development in patients expressing treatment failure, a substantial proportion of patients (27%) experienced virological re-suppression without any treatment switch, despite initial signs of virological failure. Improved adherence following intensified counselling probably accounted for the decline in viral load, as was also reported for another cohort of subtype C infected patients(11). Switching to second-line ART at first detection of viremia in these patients would lead to an unnecessary cost-increase and may negatively affect drug-toxicity profiles. Wider access to virological monitoring and resistance testing at first sign of virological failure may differentiate between lack of adherence and initial therapy failure and has recently been suggested to be cost-effective in LICs(22). An alternative and somewhat cheaper approach may be to intensify adherence counselling immediately after virological failure is established and to repeat HIV-RNA
testing soon thereafter (after 2-3 months). In case of continued virological failure, resistance testing can be done and patients should be switched to second-line ART.

As described for other African ART programmes\(^4, 11, 23-27\), the lamivudine-associated M184V and multiple NNRTI-associated mutations were most common at first detection of failure. These resistance profiles suggest that a boosted PI combined with a NRTI-backbone - commonly used as second-line ART in LICs - is a sound option.

Our data indicate that accumulation of multi-NRTI resistance mutations will seriously hamper predicted NRTI-efficacy when treatment switches are delayed. In nearly 60% of stavudine-receiving patients, tenofovir-related resistance (K65R or K70E) was ultimately present. This observation supports the suggested preferential selection of K65R in HIV-1 subtype C\(^{28}\). Apart from its unfavourable drug-toxicity profile, this cross-resistance profile between stavudine and tenofovir is of concern regarding long-term stavudine use.

Zidovudine does not seem to be clearly advantageous either, since we observed accumulation of TAMs after prolonged failure, which may confer cross-resistance to tenofovir. Nonetheless, even after prolonged viremia in the presence of drug-pressure, K65R and TAMs were never observed simultaneously, consistent with the antagonistic effects reported for these mutations\(^{29, 30}\).

Taking all this into account, tenofovir seems to be a better first-line option. This strategy is now adopted in many countries. If HIV selects K65R in the presence of tenofovir, the virus becomes hyper-sensitive to zidovudine and the selection of TAMs will be delayed. The combination of zidovudine and lamivudine may therefore still be effective in a second-line regimen.
If, after prolonged failure and accumulation of TAMs, the NRTI-backbone efficacy is severely affected, second-line therapy will be mainly dependant on the boosted PI. It remains to be seen how effective such boosted PI “mono-therapy” will be on a population level, but favourable short-term virological outcomes with such second-line regimens have been reported (31). Alternatively, composing a regimen without any NRTI, for instance by combining a boosted PI with an integrase inhibitor, may prove to be an option. To date, real monotherapy with a boosted PI can not be recommended for patients experiencing virological failure, as several clinical trials have failed to prove non-inferiority of boosted lopinavir mono-therapy as maintenance therapy (in patients who had been virologically suppressed for some time) and as no trials on boosted PI mono-therapy have been done, including patients experiencing virological failure (32, 33). Real mono-therapy with a boosted PI composing a second-line ART regimen for children is even more difficult, as tenofovir is contra-indicated in the prepubertal phase (7). Future studies will have to determine which second-line regimen will be optimal for LICs in terms of efficacy, costs and tolerability.

Two patients harboured drug-resistant HIV at baseline. Whether this observation implies that some patients were not truly treatment-naïve at baseline or whether it reflects transmission of drug-resistance (TDR) could not be specified. Future studies will need to determine the actual TDR-prevalence, using WHO threshold survey protocols (34). As two out of the four TAMs present at t1 were identified in patients with baseline resistance, excluding patients with baseline resistance would further augment the observed difference in TAM frequency between t1 and t2.

The strength of our study lies in the regular virological monitoring that was done for all patients. Consequently, a unique cohort of subtype-C infected patients with ongoing viremia during first-line ART could be defined. Moreover, the frequency of blood sample collection enabled us to gain some insight in the speed at which accumulation of drug-resistance occurs. Conversely, the
retrospective nature of our study, and the low number of patients included for genotypic analysis, are limitations of this study. Moreover, the strength of our study is restricted by the limited availability of baseline samples and the presence of some baseline resistance.

In conclusion, good virological results can be achieved in HIV-1 subtype C infected patients receiving ART in rural South Africa. However, ongoing viremia in patients experiencing virological failure leads to rapid accumulation of major drug-resistance mutations. The frequent selection of multi-NRTI resistance mutations limits future treatment options and will regularly remain unnoticed due to the limited correlation with commonly used failure criteria and irregular laboratory monitoring. These data emphasise the need to increase access to virological monitoring and to expand the antiretroviral armamentarium in LICs.

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