Original article

Low rates of nucleoside reverse transcriptase inhibitor resistance in a well-monitored cohort in South Africa on antiretroviral therapy

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Background: The emergence of complex HIV-1 drug resistance mutations has been linked to the duration of time patients are on a failing antiretroviral drug regimen. This study reports on resistance profiles in a closely monitored subtype C infected cohort.

Methods: A total of 812 participants were enrolled into the CIPRA-SA 'safeguard the household' study, viral loads were determined at 12-weekly intervals for 96 weeks. Virological failure was defined as either a <1.5 log decrease in viral load at week 12 or two consecutive viral load measurements of >1,000 RNA copies/ml after week 24. Regimens prescribed were in line with the South African roll-out programme (stavudine, lamivudine, efavirenz or nevirapine). Viral RNA was extracted from patients with virological failure, and *pol* reverse-transcriptase

PCR and sequence analysis were performed to determine drug-resistant mutations.

Results: Virological failure was observed in 83 participants on the first-line regimen during the study period, of which 61 (73%) had HIV-1 drug-resistant mutations. The M184V mutation was the most frequent (*n*=46; 65%), followed by K103N (46%) and Y181C (21%). Thymidine analogue mutations were infrequent (1%) and Q151M was not observed.

Conclusions: Drug resistance profiles were less complex than has been previously reported in South Africa using the same antiretroviral drug regimens. These data suggest that frequent viral load monitoring limits the level and complexity of resistance observed in HIV-1 subtype C, preserving susceptibility to second-line options.

Introduction

Access to antiretroviral therapy (ART) in sub-Saharan Africa has increased rapidly during the past decade, and now efforts must be put into maintaining individual patients on lifelong ART. The long-term challenges of providing ART include managing toxicities associated with extended antiretroviral (ARV) use and the development of ARV drug resistance, both of which limit future drug options available to the patient. The current ARV drug resistance patterns that have been documented in resource-limited settings show high levels of complex nucleoside reverse transcriptase inhibitor (NRTI) resistance profiles, which is likely to impair future NRTI usage [1–5]. Furthermore, the presence of

non-NRTI (NNRTI) mutations with delayed detection of ART failure could compromise the use of second-generation NNRTIs, such as etravirine.

In most resource-limited settings, immunological and virological monitoring is conducted infrequently because of cost, limited infrastructure and shortage of technical skills. In such settings, where ART failure is generally assessed through clinical staging and/or CD4+ T-cell counts, usually without viral load testing, a complex pattern of resistance has been observed. A recent study from Malawi has shown that 95% of patients failing the first-line regimen (stavudine [d4T], lamivudine [3TC] and nevirapine [NVP]) had drug resistance

[1,2]. A large proportion of these patients harboured mutations associated with cross-resistance to most NRTIs (K65R [19%], Q151M [19%] and/or thymidine analogue mutations [TAMs; 56%]), limiting the potential future use of fully susceptible NRTIs [1]. High levels of TAMs have been observed in Tanzania (28%) [6], Botswana (59%) [7], South Africa (11–32%) [3–5] and Uganda (74%) [8]. Furthermore, K65R has been observed in a high frequency, which is linked to nucleotide changes in HIV-1 subtype C [4,5,9–11]. The high level of NRTI resistance, owing to mutations K65R, Q151M and TAMs, would weaken second-line regimens containing a boosted protease inhibitor (PI) as the only active ARV. This is known to lower the barrier for selection of PI resistance [12].

In South Africa, where viral load testing is widely available as part of public-sector ART, prior to April 2010 switching to second-line therapy was only considered under South African guidelines when two consecutive viral load measurements >5,000 HIV RNA copies/ml were detected [13]. In this setting, consistent resistance data from ART centres reflect a maximum of 39% complex drug-resistant patterns (defined as the presence of either K65R and/or Q151M and/or two or more TAMs) [3–5].

Routine resistance monitoring is currently not performed within the South African national ARV treatment programme and it remains difficult in many clinics to analyse clinical and laboratory data because of the absence of linked laboratory and clinical electronic medical records. The Comprehensive International Programme of Research on AIDS in South Africa (CIPRA-SA) 'Safeguard the household' study was a randomized controlled trial of ARV-monitoring strategies in South Africa, with the primary objective of evaluating the care given by nurses versus doctors [14]. Because the first- and second-line regimens used in the study were those of the national ARV roll-out programme up until April 2010 [13], laboratory data from this study allow for a unique opportunity to examine the resistance patterns within the South African ARV roll-out programme in a well-monitored cohort. We set out to describe the resistance patterns emerging in HIV-1 subtype C infected patients in South Africa who were receiving ART in order to guide future government programmes.

Methods

Study participants

During a period of 2 years (February 2005 – January 2007), 812 HIV-1-positive participants were enrolled into the CIPRA-SA study from either Soweto in Gauteng Province or Masiphumelele in the Western Cape. Participants were ≥18 years of age, had a CD4$^+$ T-cell count <350 cells/mm^3, had no active opportunistic infections at the time of enrolment and were ART naive (excluding previous single-dose NVP [sdNVP] exposure). The participants were randomized into two arms (primary healthcare sister managed versus doctor managed), initiated on first-line ART, monitored every 3 months (viral load, CD4$^+$ T-cell, hepatic and renal function) and followed for a minimum of 96 weeks [14]. Adherence data were collected at every scheduled visit from week 4 until study completion using clinic-based pill count.

ARV treatment regimens

All patients in the CIPRA-SA cohort were given a first-line ART regimen containing d4T and 3TC, and the majority was given efavirenz (EFV) as the third drug. However, if female patients were of child-bearing age and unwilling to use two forms of contraception, NVP was prescribed instead. Lopinavir boosted with ritonavir (LVP/r) or nelfinavir (NLF) could be prescribed for women pregnant at treatment initiation. One drug substitution was permitted if drug toxicity above grade 3 was observed.

Study design

We conducted a study of resistance patterns among participants in the CIPRA-SA cohort who failed first-line treatment at two time points, at enrolment and at time of virological failure. HIV-1 drug resistance testing was conducted on all participants determined to have viral treatment failure; women who had previous sdNVP exposure to prevent peripartum mother-to-child transmission (PMTCT) were also included. Treatment failure was defined as either failure to suppress, defined as failure to achieve a 1.5 log drop in HIV viral load by week 12 on ART; or virological rebound defined as two consecutive HIV viral load measurements of >1,000 RNA copies/ml after week 24 on ART recorded more than 4 weeks apart.

The study was approved by the Human Research and Ethics Committee of the University of the Witwatersrand, Johannesburg, South Africa, and the University of Cape Town Research Ethics Committee, Cape Town, South Africa, and approval was given by the Boston University Institutional Review Board for analysis of anonymous data.

Population genotype analysis

Population-based genotyping was performed using an in-house drug resistance assay [15]. Viral RNA was extracted from 200 µl of plasma samples using the automated Roche MagNa Pure LC Analyzer and the MagNA Pure LC Total Nucleic Acid Isolation Kit (Roche, Mannheim, Germany). A 1.7 kilobase amplicon was generated by reverse-transcriptase (RT)-initiated PCR encompassing the entire protease (PR) and partial RT coding regions using primers designed from the

Table 1. Baseline characteristics at enrolment of the 812 participants and 83 virological treatment failures in the CIPRA-SA study in South Africa

Variable	No virological failure (n=729)	Virological failure (n=83)	Total (n=812)	P-value[a]
Female, n (%)	511 (70.1)	62 (74.7)	573 (70.6)	0.3833
Median age, years (IQR)	32.3 (28.0–37.2)	31.9 (28.0–36.2)	32.3 (28.0– 37.1)	0.6046
Nurse-managed care, n (%)	360 (49.4)	44 (53.0)	404 (49.8)	0.5309
First-line ART regimen				0.0012
D4T-3TC-EFV, n (%)	548 (75.2)	49 (59.0)	597 (73.5)	
D4T-3TC-NVP, n (%)	131 (18.0)	22 (26.5)	153 (18.8)	
D4T-3TC-LPVr, n (%)	46 (6.3)	9 (10.9)	55 (6.8)	
D4T-3TC-NLF, n (%)	4 (0.5)	3 (3.6)	7 (0.9)	
Study site				0.0590
Soweto, n (%)	395 (54.2)	54 (65.1)	449 (55.3)	
Masiphumelele, n (%)	334 (45.8)	29 (34.9)	363 (44.7)	
Single dose NVP exposed[b], n (%)	143 (28.0)	24 (38.7)	167 (29.1)	0.0793
CDC Stage				0.4549
A, n (%)	265 (36.4)	36 (43.4)	301 (37.1)	
B, n (%)	208 (28.5)	21 (25.3)	229 (28.2)	
C, n (%)	256 (35.1)	26 (31.3)	282 (34.7)	
Median CD4+ T-cell count, cells/mm³ (IQR[b])	167 (109–234)	147 (106–198)	164 (109–229)	0.0453
CD4+ T-cell count				0.0145
<200, n (%)	454 (62.3)	63 (75.9)	517 (63.7)	
≥200, n (%)	275 (37.7)	20 (24.1)	295 (36.3)	
Median BMI (IQR[b])	23.5 (20.7-27.3)	23.5 (21.1–26.7)	23.5 (20.8–27.2)	0.4601
Mean baseline HIV-1 RNA, log$_{10}$ copies/ml (IQR[b])	5.1 (4.6–5.6)	5.2 (4.8–5.5)	5.1 (4.6–5.6)	0.2320

[a]P-values from χ^2 test for categorical variables and t-tests for continuous variables [b]Denominator is only female. ART, antiretroviral therapy; BMI, body mass index; CIPRA-SA, Comprehensive International Programme of Research on AIDS-South Africa; D4T, Stavudine; EFV, efavirenz; IQR, interquartile range; LPVr, lopinavir/ritonavir; NLF, nelfinavir; NVP, nevirapine; 3TC, lamivudine.

consensus HIV-1 subtype C sequence available on the Los Alamos HIV Databases [16]. The amplicon was sequenced using five primers that ensure bidirectional coverage from codons 1–99 of PR and codons 1–230 of RT. Sequencing was performed with an ABI Prism 3730 Genetic Analyzer (Applied Biosystems, Foster City, CA, USA).

Data analysis

Sequences were assembled, manually edited using Sequencher version 4.5 software (Genecodes, Ann Arbor, MI, USA) and submitted to the ViroScore database, which uses the IAS-USA mutation list [17] to identify known HIV-1 drug resistance mutations associated with decreased activity of the PR and RT inhibitors. The frequency for each of these mutations was analysed by ART regimen and is presented with 95% confidence intervals. Sequences generated were subtyped using the published REGA subtyping tool version 2 [18,19].

Statistical analysis was performed using Statistical Analysis Software (SAS version 9.2, Cary, NC, USA). We compared the baseline characteristic between those who failed and those who did not using simple proportions (with 95% confidence intervals where appropriate) for categorical variables, and medians and interquartile ranges (IQRs) for continuous variables.

We compared baseline characteristics using t-tests for continuous variables and χ^2 tests for categorical variables. The difference in frequency of mutations was summarized using simple proportions and comparisons between EFV and NVP exposure were made using a χ^2 or Fisher's exact tests. A P-value of <0.05 was considered significant.

Results

Table 1 shows demographic and clinical characteristics of the cohort at enrolment. At enrolment, 517 of the 812 participants (64%) had CD4+ T-cell counts <200 cells/mm³. The median baseline CD4+ T-cell was 164 cells/mm³ and median log viral load was 5.1 copies/ml. Of participants enrolled in the study, 35% had a CDC stage C classification, defined by CD4+ T-cell count and symptomatic conditions attributed to HIV-1 infection. Seventy-one percent (573/812) of the cohort were female and 99% were of African descent. A total of 83 participants experienced virological failure on first-line ART (10.2%; 95% CI 8.3, 12.5), the majority of whom were initiated on d4T–3TC–EFV (n=49; 59%) or d4T–3TC–NVP (n=22; 27%). Twelve participants (pregnant at enrolment) were on a PI-based regimen at the time of failure (n=3; d4T–3TC–NLF and

Figure 1. Frequency of the HIV-1 antiretroviral drug resistance mutations associated with NRTI resistance in the 83 CIPRA-SA participants experiencing viral failure on ARV therapy in South Africa

Frequency, %	A62V	K65R	D67G	D67N	D67A	T69L	K70R	V75I	V118I	M184V	K219E
	1	2	1	1	1	1	1	1	1	57	1
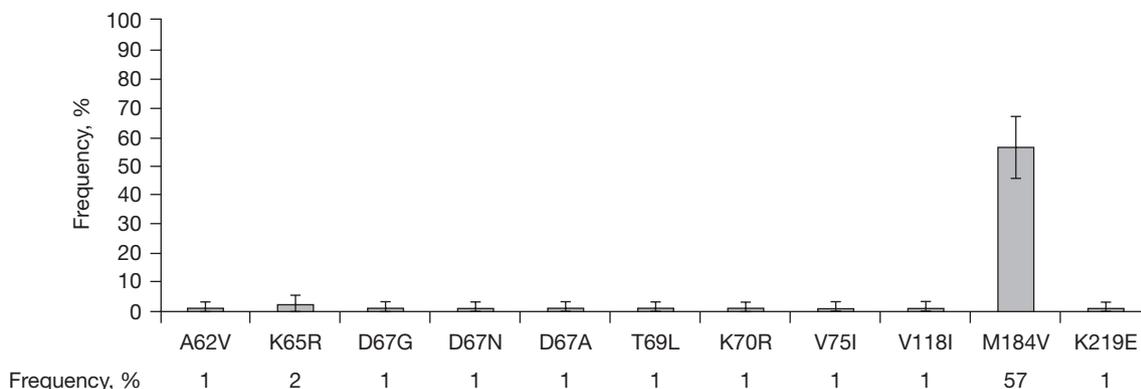
ARV, antiretroviral; CIPRA-SA, Comprehensive International Programme of Research on AIDS in South Africa; NRTI, nucleoside reverse transcriptase inhibitor.

n=9; d4T–3TC–LPV/r). The median viral load at failure was 3.9 log copies (IQR 3.5–4.8) with a median time to virological failure of 60 weeks.

Baseline characteristics showed that subjects who failed ART had lower baseline CD4+ T-cell counts (median 167 versus 147, respectively; *P*=0.045) than those who did not fail. Women who failed were more often exposed to sdNVP than were those with ART success, but this difference was not significant (39% versus 28%; *P*=0.079). Failing patients were more likely to be on a NVP-based regimen than those who did not fail (59% versus 75%; *P*=0.001). Age, gender, treatment arm, study site and baseline viral load were not related to virological failure.

Resistance

For the 83 participants experiencing virological failure, sequencing data were available for all samples at both study entry and virological failure. At virological failure known HIV-1 drug resistance mutations were observed in 73% (*n*=61) and 27% (*n*=22) had wild-type virus. The M184V mutation was the most frequent (*n*=47; 57%), followed by K103N (46%) and Y181C (21%). TAMs were infrequent (1%) and Q151M was not observed (Figure 1). Of the 83 participants, 13 (16%) failed to suppress viral load and 70 (84%) experienced viral rebound.

Of the 13 participants who failed to suppress viral load, none had mutations associated with resistance at study enrolment, whereas at failure 4 (31%; 95% CI 11, 59) had mutations. The most frequent mutation was K103N (*n*=3; 23%) followed by V106A/M (*n*=2; 15%). The Y181C and M184V mutations occurred in one patient each.

Baseline sequencing revealed that of the 70 subjects who experienced viral rebound, 5 (7%) were found to have resistance at study entry and 57 (81%) had resistance at failure. At time of failure the following NRTI mutations were observed: A62V (*n*=1; 1%), K65R (*n*=2; 3%), D67G/N (*n*=2; 3%), T69L (*n*=1; 1%), K70R (*n*=1; 1%), V75I (*n*=1; 1%), M184V (*n*=46; 66%) and K219K (*n*=1; 1%). The M184V mutation was the most prevalent mutation, occurring in 46 patients experiencing viral rebound (66%; 95% CI 54, 76; Figure 2) followed by the K65R mutation. Only one patient had TAMs and Q151M was not observed. A total of 13 (19%) of the participants had no mutations associated with resistance (Figure 2).

Of the five with resistance at enrolment, no NRTI resistance was observed, two had resistance to NNR-TIs (K103N *n*=1; V106M, K103N *n*=1), two had protease resistance (M46I *n*=1, M46L *n*=1) and one had both protease and NNRTI resistance (M46V, K101E, G190A). All three participants with NNRTI mutations were female, but only one reported previous sdNVP exposure to prevent two separate cases of MTCT (1 and 36 months prior to study entry). Only one of these patients with baseline resistance (M46I) would have been fully susceptible to the regimen they were prescribed (d4T, 3TC and EFV). The participants who were not completely susceptible to their regimens suppressed for an average of 11 months, whereas the patient who was fully susceptible suppressed for 30 months.

Resistance by regimen

Of the 83 patients experiencing virological failure, different mutation patterns were observed in the 71

Figure 2. HIV-1 antiretroviral resistance patterns in 70 participants experiencing viral rebound on first-line therapy in the CIPRA-SA study in South Africa.
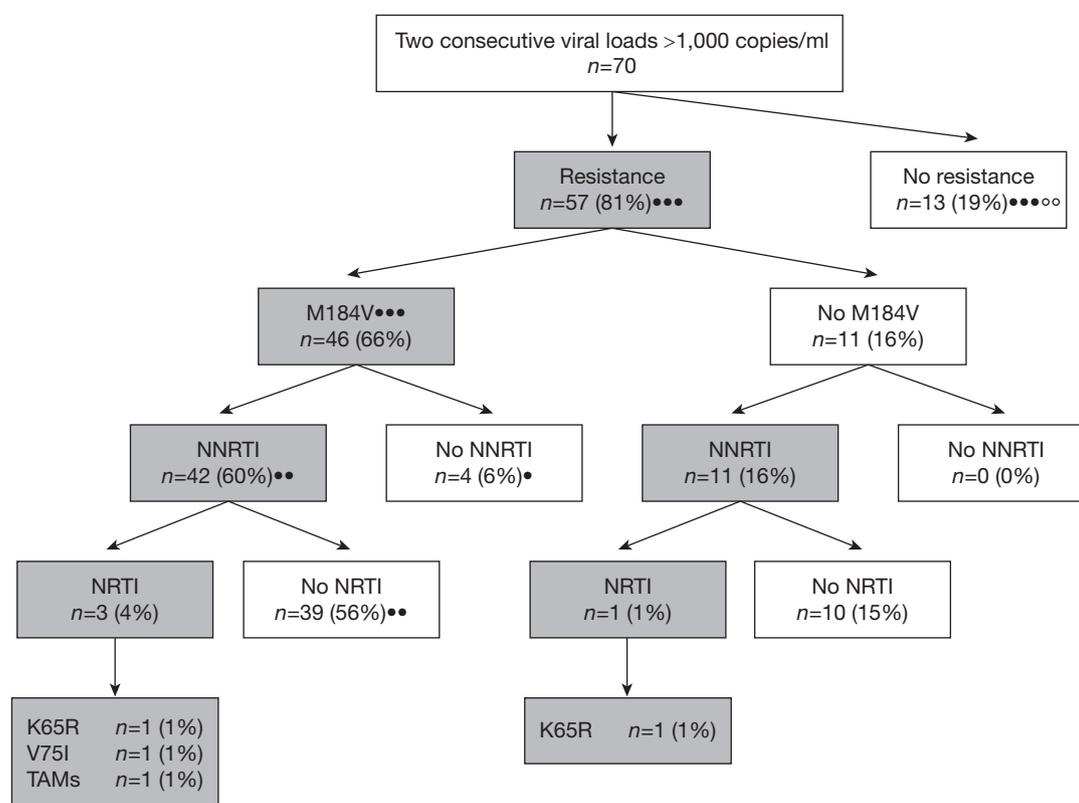
Participants on a protease inhibitor-based regimen are represented by open circles; participants also exposed to single-dose nevirapene to prevent mother-to-child-transmission are represented by closed circles. CIPRA-SA, Comprehensive International Programme of Research on AIDS in South Africa; NRTI, nucleoside reverse transcriptase inhibitor; NNRTI, non-nucleoside reverse transcriptase inhibitor; TAMs, thymidine analogue mutations.

participants accessing either an EFV- (*n*=49) or NVP-containing (*n*=22) regimen (Figure 3). Of the patients failing an EFV or NVP regimen, 27% (*n*=13) and 18% (*n*=4), respectively, had no NNRTI mutation present at virological failure. The Y181C and V106A mutations only occurred in participants accessing a failing NVP-containing regimen (41% and 9%, respectively). Both the K103N and V106M mutations occurred more frequently in EFV- than NVP-exposed patients, but differences were not significant (51% versus 32%, *P*=0.0064 and 16% versus 9%, *P*=0.1345, respectively). EFV selected for a wider range of mutations in the RT region than did NVP (Figure 3).
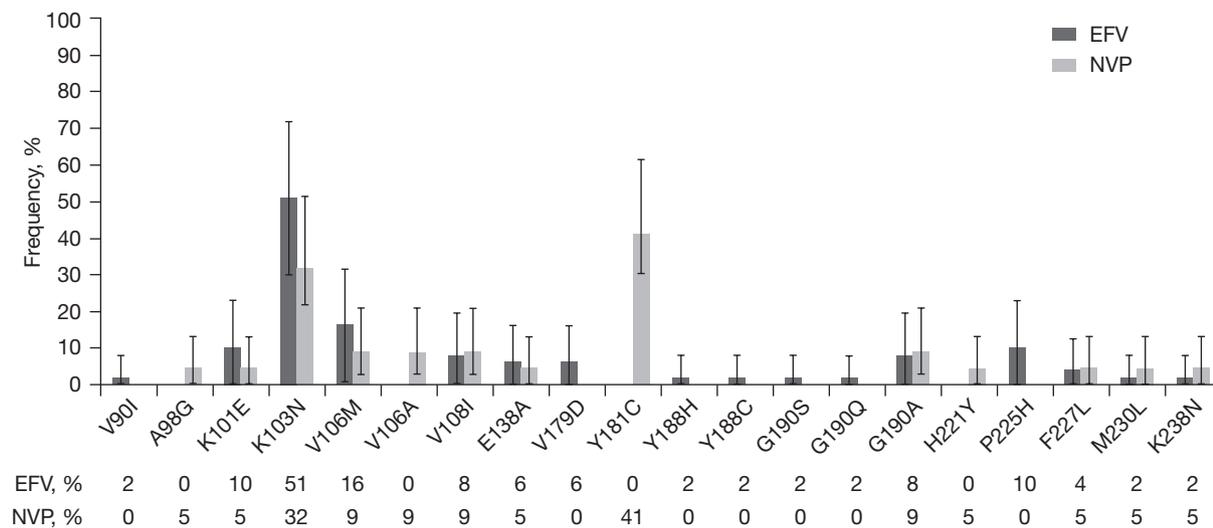
Of the 12 patients failing a PI-containing regimen, 58% (*n*=7) had no resistance at virological failure. Nine of the 12 were accessing a LPV/r and 3 were accessing NLF-based regimens. Protease resistance was only observed in one patient accessing LPV/r; however, the M46L mutation was present at enrolment. For the remaining nine participants failing a LPV/r regimen, M184V was

observed in one patient and K103N, V106A and Y188C in another female patient (with no previous sdNVP exposure reported). All three patients failing the NLF-based regimen had the M184V mutation and the two with prior sdNVP exposure also had NNRTI mutations (K103N, V106M *n*=1; K103N, Y188H *n*=1).

Discussion

This is the first study describing the HIV-1 drug resistance mutation patterns in HIV-1 subtype C infected individuals in a well-monitored cohort, using ARV drug regimens that mirrored that of the South African national roll-out programme [13]. Of the 83 patients defined as meeting the virological failure criteria of the study, 73% (*n*=61) had an NRTI and/or NNRTI resistance mutation. The most frequently observed mutations were M184V (57%), K103N (46%) and Y181C (21%). The mutation K65R and TAMs were observed infrequently, and the Q151M mutation was not present at

Figure 3. Distribution of NNRTI mutations in patients failing first-line antiretroviral regimens in the CIPRA-SA study in South Africa

	V90I	A98G	K101E	K103N	V106M	V106A	V108I	E138A	V179D	Y181C	Y188H	Y188C	G190S	G190Q	G190A	H221Y	P225H	F227L	M230L	K238N
EFV, %	2	0	10	51	16	0	8	6	6	0	2	2	2	2	8	0	10	4	2	2
NVP, %	0	5	5	32	9	9	9	5	0	41	0	0	0	0	9	5	0	5	5	5

First-line antiretroviral regimens contained efavirenz (EFV; *n*=49) or nevirapine (NVP; *n*=22). The error bars represent the confidence intervals. CIPRA-SA, Comprehensive International Programme of Research on AIDS in South Africa; NNRTI, non-nucleoside reverse transcriptase inhibitor.

all, thereby preserving the use of zidovudine, didanosine or tenofovir in second-line regimens.

The mutation patterns were less complex than those reported in published data from the region [1,3–5]; K65R (3%) was considerably lower than that observed in first-line failures in previously published data from Malawi [1] and South Africa [5], which showed M184V present in up to 81% of participants and K65R present in up to 19%. Furthermore, only one participant in this study harboured TAMs, in complete contrast to all other published studies from the region, which report levels of 23–56% [1,3–5], and the Q151M mutation was not observed. The differences in resistance levels observed could be attributed to several different factors. Firstly, stringent monitoring and switch criteria (switch based on viral load >1,000 RNA copies/ml versus a higher threshold of >5,000 RNA copies/ml, or switch based on clinical/immunological criteria) could prevent prolonged ART failure. A comparison of viral loads at failure between this study and others in the region [3–5] indicated that the median viral load of this cohort (3.95 log copies/ml) was half a log lower than those in the other studies (4.29, 4.88 and 4.43 log copies/ml, respectively). Secondly, the increased frequency of CD4 and viral load monitoring, namely, 3-monthly compared with 6-monthly in the South African national programme until April 2010, could decrease ART failure. Both of these factors are in line with studies from developed countries that have linked duration of treatment

failure to frequency and complexity of mutation profiles [20,21].

The second most frequent NRTI mutation observed was K65R (3%), which is uncommon in HIV-1 subtype B infected patients receiving d4T [22]. This finding is similar to those in the region [1,4,5] and in *in vitro* cell culture studies [9,23,24]. The increased frequency of K65R has been linked to nucleotide changes in the sequence prior to codon 65 in HIV subtype C [10,11]. The K65R mutation results in broad cross-resistance to NRTIs [17] and has consequences for the subsequent use of most NRTIs, especially tenofovir in second-line regimens, possibly making it better suited to first-line regimens. Furthermore, the presence of K65R might have implications for tenofovir usage in pre-exposure prophylaxis and further investigation is required into transmission and fitness of viruses with K65R.

NVP and EFV were used by participants in this study and it was observed that EFV selected for a wider range of resistance mutations in the RT area investigated than did NVP, although, again, the numbers were small. The K103N mutation was the most frequent NNRTI mutation observed (41%). NVP uniquely selected for Y181C (41%) and V106A (9%) and both the K103N and V106M mutations were more frequent in participants accessing EFV than in those accessing NVP. These NNRTI mutation patterns are the same as those observed in the South African public sector programme [5] with K103N being the most prevalent. The difference in mutations selected

by EFV and NVP could have an impact on the use of second-generation NNRTIs, such as etravirine (ETR) in future second- or third-line regimens. For example, the use of NVP can lead to the emergence of Y181C, which results in a significant reduction in the susceptibility to ETR, whereas the emergence of K103N from the use of EFV does not affect drug susceptibility to second-generation ETR.

No HIV drug resistance mutations were present in 27% of subjects. This could be a result of poor adherence, which was not addressed in this paper, especially in the group of participants that did not achieve a 1.5 log reduction in viraemia by week 12. This finding substantiates the use of drug-resistance testing after first-line failure to decrease the number of patients who are switched unnecessarily to more expensive second-line regimens. Instead of switching these patients without mutations to the second-line regimen, they should undergo intensive adherence counselling.

In conclusion, this study has shown that the complexity of drug-resistance patterns in resource-limited settings can be greatly reduced when both strict and frequent virological monitoring are used to detect ART failure. This underscores the importance of using routine viral load testing and strict switching criteria to reduce the duration on a failing regimen and limit the development of complex resistance patterns. This strategy will preserve future treatment options for either second- or third-line ARV treatment regimens. Furthermore, the use of HIV-1 drug resistance testing after first-line failure will reduce the number of unnecessary switches to more expensive second-line regimens.

Disclosure statement

The authors declare no competing interests.

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