

# Drug Resistance and Viral Tropism in HIV-1 Subtype C-Infected Patients in KwaZulu-Natal, South Africa: Implications for Future Treatment Options

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**Background:** Drug resistance poses a significant challenge for the successful application of highly active antiretroviral therapy (HAART) globally. Furthermore, emergence of HIV-1 isolates that preferentially use CXCR4 as a coreceptor for cell entry, either as a consequence of natural viral evolution or HAART use, may compromise the efficacy of CCR5 antagonists as alternative antiviral therapy.

**Methods:** We sequenced the *pol* gene of viruses from 45 individuals failing at least 6 months of HAART in Durban, South Africa, to determine the prevalence and patterns of drug-resistance mutations. Coreceptor use profiles of these viruses and those from 45 HAART-naïve individuals were analyzed using phenotypic and genotypic approaches.

**Results:** Ninety-five percent of HAART-failing patients had at least one drug-resistant mutation. Thymidine analog mutations (TAMs) were present in 55% of patients with 9% of individuals possessing mutations indicative of the TAM1 pathway, 44% had TAM2, whereas 7% had mutations common to both pathways. Sixty percent of HAART-failing subjects had X4/dual/mixed-tropic viruses compared with 30% of HAART-naïve subjects ( $P < 0.02$ ). Genetic coreceptor use prediction algorithms correlated with phenotypic results with 60% of samples

from HAART-failing subjects predicted to possess CXCR4-using (X4/dual/mixed viruses) versus 15% of HAART-naïve patients.

**Conclusions:** The high proportion of TAMs and X4/dual/mixed HIV-1 viruses among patients failing therapy highlight the need for intensified monitoring of patients taking HAART and the problem of diminished drug options (including CCR5 antagonists) for patients failing therapy in resource-poor settings.

**Key Words:** coreceptor use, viral tropism, antiretroviral drug resistance, HAART-failing patients, HAART-naïve patients

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## INTRODUCTION

HIV/AIDS is the leading cause of death in sub-Saharan Africa and South Africa has the highest number of HIV infections worldwide.<sup>1</sup> HIV-1 subtype C (HIV-1C) is responsible for the majority of infected individuals in South Africa and worldwide.<sup>2</sup> Access to highly active antiretroviral therapy (HAART) in South Africa has increased dramatically since the launch of the Operational Plan for Comprehensive HIV and AIDS Care and Treatment in 2003.<sup>3</sup> However, antiretroviral drug coverage in sub-Saharan Africa remains low and it was estimated that in 2009, only 37% of patients eligible for treatment according to the World Health Organization guidelines were receiving it.<sup>4</sup>

Despite the suppression of HIV-1 plasma viral load by HAART to undetectable levels, viral transcription persists<sup>5,6</sup> and this can lead to the emergence and persistence of drug resistance, which has significant public health implications. Most developing countries rely on clinical or immunologic algorithms to monitor the effectiveness of HAART,<sup>7</sup> which may result in a delay in the switching of failing HAART regimens. The delay in the switching of failing HAART regimens in the developing world, where World Health Organization guidelines recommend the use of two nucleoside reverse transcriptase inhibitors (NRTIs) and one nonnucleoside reverse transcriptase inhibitor (NNRTI) as first-line therapy, may in turn result in the accumulation of thymidine analog mutations (TAMs), which are associated with broad cross-resistance and may therefore limit the options available for alternative regimens. Recent studies have highlighted this emerging problem.<sup>8–11</sup>

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In addition to concerns regarding the emergence of TAMs in resource-poor settings, there are suggestive data that individuals failing HAART may have a higher proportion of CXCR4-using viruses compared with antiretroviral-naïve patients even after controlling for the level of immunodeficiency between the groups.<sup>12,13</sup> CXCR4 (X4) and dual-tropic (R5X4/dual) viruses are associated with rapid disease progression and emerge in the late chronic phase of disease in a significant proportion of patients.<sup>14,15</sup> However, the switch to X4 viruses appears to be significantly less common for HIV-1C, even in late stages of disease.<sup>16–20</sup> The predominant use of CCR5 by HIV-1C could be interpreted to suggest that CCR5 antagonists would be more efficacious for this subtype. Therefore, characterization of viral tropism in HIV-1C could help inform whether CCR5 antagonists should be used as salvage therapy in patients failing current widely used regimens or as part of first-line/early regimens for maximum benefit.

We investigated the drug resistance mutational pathways and factors associated with failure of HAART among HIV-1C-infected patients in a setting where monitoring relies mainly on clinical and immunologic algorithms. Furthermore, we determined coreceptor use profiles of HIV-1C viruses from individuals initiating or failing HAART to assess the usefulness of CCR5 antagonists as first-line or salvage therapy. We also explored the accuracy of *env* sequence-based genotypic predictive algorithms in assessing the prevalence of R5 and CXCR4-using viruses in these patients.

## MATERIALS AND METHODS

### Study Participants

Study participants were recruited from the Sinikithemba outpatient HIV/AIDS clinic at McCord Hospital in Durban, South Africa. Patients were included in the antiretroviral therapy (ARV)-naïve cohort if they were at least 18 years of age, had a known HIV infection, and had no history of HAART (the use of single-dose nevirapine for the prevention of mother-to-child HIV transmission was not an exclusion criterion). Patients who met these criteria, had CD4+ T-cell counts 200 cells/mm<sup>3</sup> or less, or displayed AIDS-defining clinical features according to World Health Organization staging irrespective of CD4 counts or viral loads were recruited into this study. Patients were included in the ARV-failing arm if they were at least 18 years of age, had a known HIV infection, HIV-1 RNA load of 5,000 copies/mL or greater, and had at least 6 months of uninterrupted HAART. HAART-failing patients were also recruited into the study if they were clinically assessed to be failing therapy irrespective of viral loads or CD4+ T-cell counts. All study participants gave written informed consent and the study was approved by all participating Institutional Review Boards.

### Sample Collection, Viral Load, and CD4 Measurement

CD4+ T-cell counts were determined from fresh blood from all participants by standard flow cytometry on a FACSCalibur (Becton-Dickinson, Franklin Lakes, NJ) according to the

manufacturer's instructions. Plasma viral loads were measured using the COBAS AmpliPrep/COBAS Amplicor HIV-1 Monitor Test, Version 1.5 (Roche Diagnostics, Rotkreuz, Switzerland).

### Genotypic Resistance Testing

Genotypic resistance testing was done from plasma samples using the Viroseq HIV-1 Genotyping System (Celera Diagnostics, Alameda, CA) as directed by the manufacturer.

### Phenotypic Coreceptor Analysis

Coreceptor use of viruses from patient plasma samples was determined using the enhanced sensitivity Trofile coreceptor tropism assay (Monogram Biosciences Inc, South San Francisco, CA).<sup>21,22</sup> The Trofile assay is a commercial, standardized cell-based approach for determination of coreceptor use by plasma-derived HIV-1 envelope proteins.<sup>23,24</sup>

### Envelope Sequence Analysis

cdNA synthesis, envelope amplification, and cloning were done as previously described.<sup>25</sup> Full-length *env* from 20 ARV-failing patients and 20 ARV-naïve patients was cloned into the pcDNA3.1D/V5-His-TOPO vector (Invitrogen, Carlsbad, CA). Sequencing was done using the ABI PRISM Big Dye Terminator Cycle Sequencing Ready Reaction kit Version 3.1 (Applied Biosystems, CA). Sequences were assembled and edited using Sequencher 4.8 (Ann Arbor, MI) and aligned with Mega 4.<sup>26</sup> Phylogenetic trees were constructed in Paup 4.0 (Sunderland, MA) and visualized using Treeview 1.6.6 (Glasgow, Scotland). Coreceptor use was predicted using various publicly available or published sequence-based predictive algorithms.<sup>27–30</sup>

### Statistical Analysis

All statistical analysis was done using Graph Pad Prism 5. Factors associated with tropism were assessed using unpaired *t* test, Fisher exact test, and logistic regression analysis.

### Nucleotide Sequence Accession Numbers

The sequence data obtained from this study have been submitted to GenBank and are available under accession numbers GU080160 to GU080199.

## RESULTS

### Demographic and Clinical Characteristics

Forty-five HAART-naïve and 45 HAART-failing patients were recruited. Patient demographic and clinical data are summarized in Table 1. At the time of analysis, HAART-naïve patients had a lower median CD4+ T-cell count (123 cells/mm<sup>3</sup>) than HAART-failing (174 cells/mm<sup>3</sup>) subjects ( $P = 0.036$ ). However, the median CD4+ T-cell count of HAART-naïve patients was higher than the nadir median CD4+ T-cell count (57 cells/mm<sup>3</sup>) ( $P = 0.0004$ ) of HAART-failing patients. HAART-naïve patients had a significantly higher median plasma viral load of 44,042 copies/mL compared with 6653 copies/mL for HAART-experienced participants ( $P = 0.001$ ). For patients failing treatment, the median duration on therapy was 29 months.

**TABLE 1.** Patient Information

Patient Characteristic	ARV-Experienced Patients Failing Treatment (n = 45)	ARV-Naïve Patients (n = 45)	P
Age, median years (Q1–Q3)	36 (24–51)	36 (20–78)	0.65
Gender: Female	28 (65%)	27 (60%)	
Black race	45 (100%)	45 (100%)	
CD4 cell count, median cells/mm <sup>3</sup> (Q1–Q3)			
Current	174 (9–718)	123 (8–660)	0.036
Nadir	57 (3–197)		0.0004
Viral load, median copies/mL	6, 653 (225–220,010)	44,042 (1,702–1,167,759)	0.0010
Current treatment regimen:			
Regimen 1A (d4T, 3TC, EFV)	23 (51.1%)		
Regimen 1B (d4T, 3TC, NVP)	2 (4.4%)		
ZDV, d4T, ddI, NVP	1 (2.2%)		
ZDV, d4T, 3TC, EFV	2 (4.4%)		
ZDV, 3TC, EFV	13 (29.0%)		
ZDV, 3TC, NVP	4 (8.9%)		

3TC, Lamivudine; ARV, antiretroviral therapy; AZT, Zidovudine; d4T, Stavudine; ddI, Didanosine; EFV, Efavirenz; NVP, Nevirapine; Q, quartile.

### Genotypic Drug Resistance Typing

Drug resistance results were obtained for 43 of the 45 HAART-failing patients. Resistance testing was also performed for 10 HAART-naïve patients. The only drug resistance mutation observed in the HAART-naïve individuals was in one patient with the NNRTI-associated E138A mutation. Three HAART-naïve patients had minor protease inhibitor resistance mutations, one patient with mutations L10V and T74S/T, another had mutation A71T, and the third patient had the T74S/T mutation. Of the 45 HAART-failing patients, 51.1% were on South African national treatment guidelines Regimen 1A (Stavudine [d4T], Lamivudine [3TC], and Efavirenz [EFV]); 4.4% were on Regimen 1B (d4T, 3TC, and Nevirapine [NVP]); 29% were on Zidovudine (AZT), 3TC and EFV; 8.9% were on AZT, 3TC, and NVP; 2.2% were on AZT, d4T, Didanosine (ddI), and NVP; and 4.4% were on AZT, d4T, 3TC, and EFV. Twenty-seven (60%) of the patients were on previous ARV therapy as detailed in Table 1.

The specific drug resistance mutations detected in HAART-failing patients and their frequencies are shown in Figure 1. Mutations to all three major classes of drugs were noted. Forty-one of the 43 (95%) ARV-failing patients possessed at least one drug resistance mutation. Ninety-one percent of patients had at least one drug resistance mutation against two classes of drugs (NRTI and NNRTI). Nineteen percent had at least one resistance mutation against all three classes of drugs (NRTI, NNRTI, and protease inhibitors). For protease inhibitor, only one minor mutation (T74S) was present in 9 (21%) of the HAART-failing patients (data not shown).

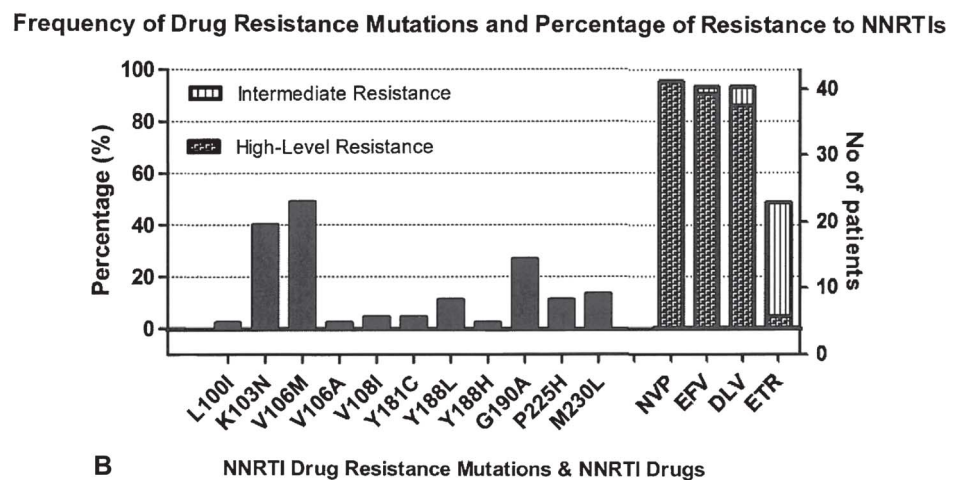
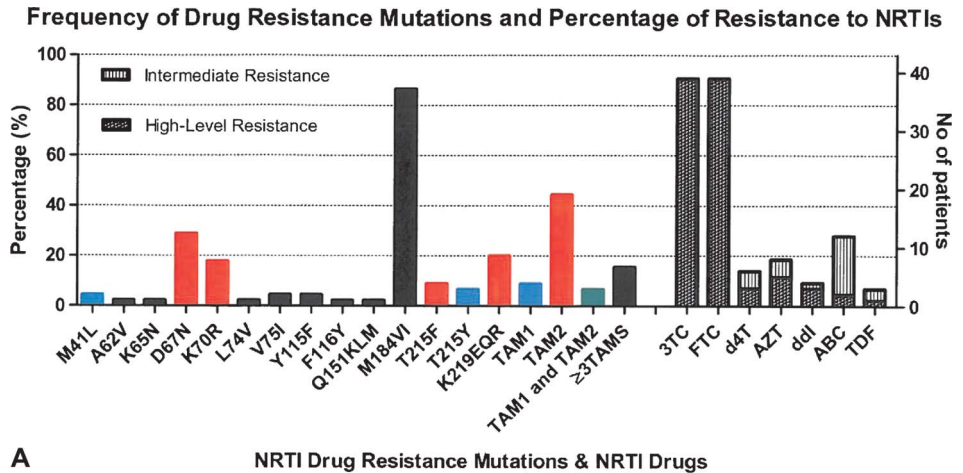
M184V/I, present in 87% of HAART-failing patients, was the most common NRTI mutation detected. TAMs were detected in 55% of patients. The TAM1 pathway NRTI mutations M41L and T215Y, associated with intermediate- to high-level resistance to AZT and d4T and low level resistance to ddI, Abacavir, and TDF,<sup>31</sup> were present in approximately 9% of patients. Neither an insertion at codon 69 nor the L210W mutation, which is also indicative of the TAM1 pathway, was noted. The TAM2 pathway mutations present were D67N, K70R, T215F, and K219E/Q/R. Forty-four percent of patients had TAM2 pathway mutations. Seven percent of patients possessed both TAM1 and TAM2 mutations and 16% had three or more TAMs.

Approximately 91% of patients had resistance to 3TC and Emtricitabine, and 19% had high- or intermediate-level resistance to AZT as defined by the Stanford database resistance scores (<http://hivdb.stanford.edu/>) (see also Figure 1 legend). Fourteen percent had high- or intermediate-level resistance to d4T, whereas 9% had high- or intermediate-level resistance to ddI. High- or intermediate-level resistance to Abacavir was noted in 28% of patients, whereas only 7% displayed high- or intermediate-level resistance to TDF (Fig. 1A).

NNRTI mutations noted are summarized in Figure 1B. The most common NNRTI resistance mutation was V106M, found in 49% of HAART-failing participants. The K103N (40%) and G190A (27%) mutations were also relatively common but no G190S mutations were present in any of the patients. V106M and K103N both cause high-level resistance to three of the four NNRTI: nevirapine, delavirdine, and EFV but has no effect on etravirine (ETR). G190A causes high-level resistance to nevirapine, intermediate resistance to EFV, and low-level resistance to ETR. This mutation also increases susceptibility to delavirdine.<sup>32</sup> Interestingly, the M230L mutation, associated with high-level resistance to ETR and uncommon (less than 5%) in both Subtype B and C NNRTI-failing patients, occurred at an unusually high frequency of 13.3% in this cohort. Ninety-five percent of patients had mutations associated with high-level resistance to nevirapine; 93% had high-/intermediate-level EFV resistance mutations. Ninety-three percent displayed high- or intermediate-level resistance to delavirdine with 49% displaying high-/intermediate-level resistance to ETR (Fig. 1B).

Recent studies in southern Africa have highlighted the growing problem of TAMs in patients receiving the World Health Organization or national antiretroviral programs recommended first-line therapy.<sup>9,10,33</sup> We hypothesized that these mutations may be increasing as antiretroviral rollout accelerates accompanied by mainly clinical and immunologic-based monitoring of treatment. We therefore compared the proportion and patterns of TAM mutations observed in our study, in which patients had been treated for a median of 29 months, with data reported from an earlier study from the same healthcare facility in patients (median treatment duration of 11 months) also following the South African national antiretroviral treatment and monitoring guidelines. We found that 55% of individuals failing therapy had TAM mutations compared with 32% reported in the earlier study<sup>10</sup> and there was a nonsignificant trend in the present study for association of TAMs with duration of treatment ( $P = 0.08$ ) (data not shown).

**FIGURE 1.** Frequency of drug resistance mutations and thymidine analog resistance mutations (TAMs). (A) The frequency of NRTI resistance mutations, thymidine analog mutation frequencies, and number of patients displaying high- and intermediate-level resistance to specific NRTIs and (B) the frequency of NNRTI resistance mutations and the number of patients showing resistance to specific NNRTIs. According to the Stanford drug resistance database algorithms for interpretation of resistance, high-level resistance refers to a genotypic pattern in which isolates have the maximum level of in vitro drug resistance and/or patients infected with isolates having similar genotypes display little or no virologic response to treatment with the drug. In low-level resistance, virus isolates have partial in vitro drug susceptibility and/or patients with viruses of this genotype may have a suboptimal virologic response to treatment compared with the treatment of a wild-type virus. Intermediate resistance suggests a degree of drug resistance greater than low-level resistance but lower than high-level resistance. NRTI, nucleoside reverse transcriptase inhibitor; NNRTI, nonnucleoside reverse transcriptase inhibitor.



## Coreceptor Use

### Phenotypic Coreceptor Analysis

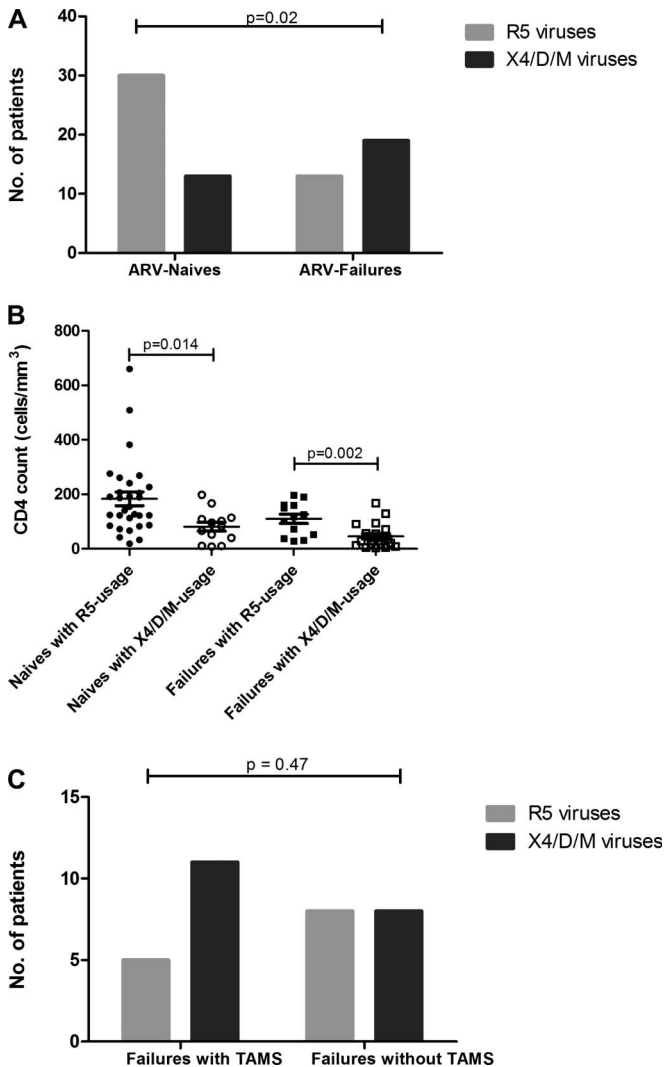
Only 83 of 90 samples were available for phenotypic coreceptor analysis of which 75 samples (32 from ARV-experienced patients failing treatment and 43 from ARV-naïve patients) yielded reportable data from the Trofile coreceptor tropism assay. Overall, 31 of 75 (41%) were dual/mixed viruses, 43 of 75 (57%) were CCR5-using, and only one (1%) was exclusively CXCR4-using. Of the 43 ARV-naïve patients, 30 (70%) possessed R5 viruses compared with 13 (30%) with dual/mixed viruses. No ARV-naïve patients exhibited exclusive X4 viruses. Among the 32 ARV-experienced patients failing treatment, 13 (41%) possessed R5 viruses, 18 (56%) had dual/mixed infections, whereas one patient (3%) had X4-only viruses (Fig. 2A). Thus, patients failing treatment had a higher percentage (59%) of X4/dual/mixed viruses compared with ARV-naïve patients with 30%; ARV-naïve patients had a higher proportion of R5 viruses (70%) compared with patients failing therapy with 41% ( $P = 0.02$ ).

We then sought to determine if there was a relationship among CD4 counts, viral loads, and viral tropism. Patients with

X4/dual/mixed viruses had significantly lower nadir CD4<sup>+</sup> T-cell counts compared with those with R5 viruses in both the ART-naïve and ART-failing groups with significant  $P$  values of 0.014 and 0.002, respectively. In logistic regression analysis, lower CD4 counts ( $P = 0.0004$ ) but not age of the patient ( $P = 0.29$ ) or duration on HAART ( $P = 0.95$ ) treatment was a significant predictor of X4/dual/mixed infections. We also investigated whether patients with TAMs were more likely to harbor dual/mixed/X4 viruses. Eleven of 16 (69%) HAART-failing patients with TAMs had X4/dual/mixed viruses and five (31%) had CCR5-using viruses compared with the respective proportions of 50% dual/mixed/X4 versus 50% R5 among patients without TAMs ( $P = 0.47$ ) (Fig. 2C).

### Genotypic Analysis of the *env* Gene

HIV-1 envelope sequence determines coreceptor use.<sup>29,34-37</sup> We therefore next sought to assess the extent to which viral tropism could be predicted by *env* sequence characteristics. We randomly selected 20 virologically failing and 20 ARV-naïve patients and analyzed full-length *env* sequences for predictive coreceptor use profiles.



**FIGURE 2.** Relationship of viral tropism of ARV-naive and ARV-failing patients. (A) Frequency of X4/dual/mixed- and R5-using viruses in patients failing treatment and treatment-naive individuals. Bar graph indicating results from the Trofile assay comparing the proportion of patients with R5 viruses with those harboring X4/dual/mixed viruses in HAART-naive versus HAART-failing patients ( $P = 0.02$ , Fisher exact test). (B) CD4 counts and coreceptor use in HAART-naive patients and HAART-failing patients. Dot graph indicating results from Trofile assay. ARV-naive patients with X4/D/M viruses had a lower CD4 count than ARV-naive patients with R5 viruses ( $P = 0.014$ , unpaired  $t$  test). ARV-failing patients with X4/D/M viruses had a lower CD4 count than ARV-failing patients with R5 viruses ( $P = 0.02$ , unpaired  $t$  test). For ARV-failing patients, nadir CD4 counts are represented. (C) Association of TAMs in X4/D/M viruses. Bar graph indicates trofile assay results. D/M indicates dual/mixed viruses. ART, antiretroviral therapy; HAART, highly active antiretroviral therapy; TAMs, thymidine analog mutations.

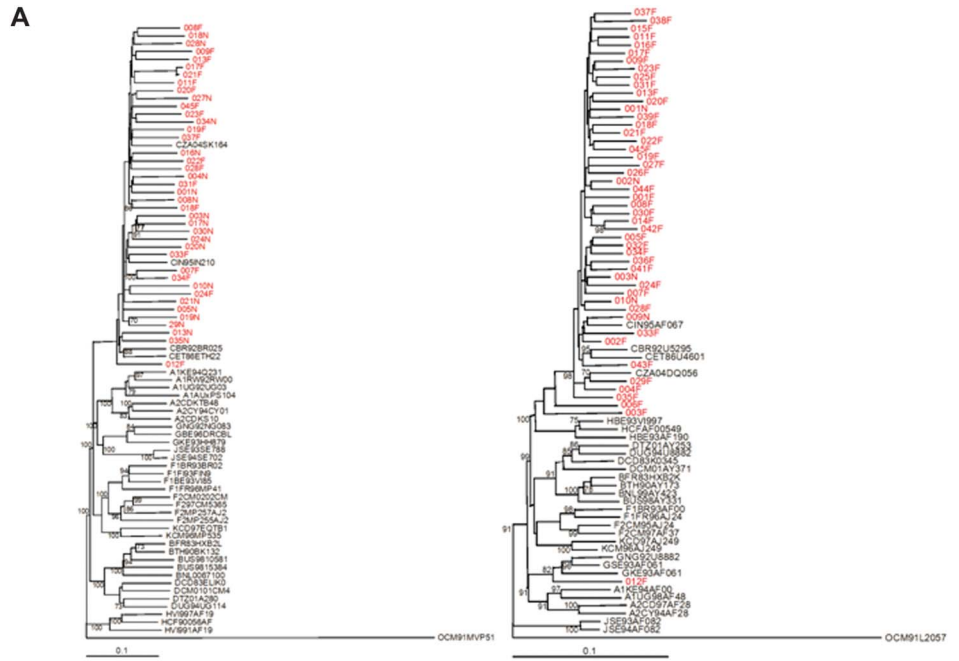
All 40 full-length *env* clones analyzed phylogenetically clustered with HIV-1C references (Fig. 3A, left panel). Phylogenetic analysis of the *pol* region also showed that all the patients clustered with HIV-1C with the exception of 704MC012F, which

is a recombinant of subtypes A and G; 704MC003F, which is a recombinant of Subtypes A and C; and 704MC006F, which is a recombinant of Subtypes C and J as confirmed using Simplot (Fig. 3A, right panel). The *env* V3 loop sequences from HAART-naïve and failing participants are shown in Figure 3B. The overall V3 consensus sequence generated for ARV-naïve patients (right panel) had two more amino acids than the consensus sequence generated for ARV-failing patients (left panel). Thirteen (65%) of failures and 17 (85%) of ARV-naïve patients had a V3 region consisting of 35 amino acids consistent with the consensus Subtype C sequence. Thirty-one of 40 (78%) clones analyzed contained the consensus subtype C GPGQ crown motif sequence.

We next investigated the reliability of V3 loop sequence-based predictive algorithms against the phenotypic results obtained using the Trofile assay. Coreceptor prediction genotypic methods evaluated here included the 11/25 rule, the overall net V3 charge, the Subtype C-specific position specific scoring matrix (C-PSSM), a web-based coreceptor prediction tool (<http://indra.mullins.microbiol.washington.edu/pssm/>), and another web-based coreceptor prediction tool, Geno2Pheno (<http://coreceptor.bioinf.mpi-inf.mpg.de/>).<sup>27–30</sup> We also investigated whether a combined algorithm (combining three of these four methods) would provide a better correlate of the phenotype data. Overall, the combined algorithm correlated with the Trofile assay results in 87% of cases, compared with 81% for C-PSSM, 78% for the 11/25 rule and 75% for the V3 net charge method and 84% for Geno2Pheno (Table 2). The combined algorithm and the 11/25 rule correctly identified 90% of R5 sequences, C-PSSM correctly predicted 85%, and the V3 net charge method predicted 71% of R5 viruses, whereas Geno2Pheno was accurate for 86% of R5 cases. In contrast, the Geno2Pheno method was the best in accurately predicting X4/dual/mixed at 82%, V3 overall charge method accurately predicted 81% X4/dual/mixed, the combined algorithm was at 80%, C-PSSM 72%, and the 11/25 rule correctly predicted only 55% of X4/dual/mixed viruses. Sensitivity of these methods was also determined using the Trofile assay as the gold standard. The Geno2Pheno and V3 charge prediction methods were 82% sensitive in predicting X4/dual/mixed virus variants followed by the combined algorithm (80%), C-PSSM (73%), and 11/25 rule (46%).

## DISCUSSION

Monitoring the emergence and patterns of antiretroviral drug resistance is crucial for the success and sustainability of treatment programs. Moreover, as new drugs become available, there is a growing need to better characterize viruses from both drug-naïve and virologically failing patients to better understand the suitability of these new drugs either as additional components of the current regimens or as salvage therapy. In this study, we investigated the prevalence and pattern of drug resistance mutations in a cohort of HIV-1C-infected individuals failing therapy. In addition, because it has previously been suggested that suboptimal ARV or certain classes of drugs may select for the more virulent X4 virus variants,<sup>13</sup> we sought to identify correlates of viral tropism in HAART-naïve and therapy-experienced virologically failing patients.



**FIGURE 3.** Neighbor-joining phylogenetic trees. (A) Left panel: neighbor-joining phylogenetic tree constructed from the *env* gene sequences. All clones highlighted in red cluster closely with subtype C. Right panel: neighbor-joining phylogenetic tree constructed from the *pol* gene sequences. All patient samples highlighted in the red cluster together with the Subtype C reference with the exception of 704MC012F, which clusters with Subtype G. Bootstrap values more than 70% are shown. (B) Alignment of V3 sequences of clones of ARV-failing and ARV-naïve patients. The panel on the left indicates V3 sequences in patients failing treatment and the panel on the right indicates V3 sequences in ARV-naïve patients. The crown motif for each sequence is highlighted in red. All sequences from viruses determined to be X4/dual/mixed by the Trofile assay are highlighted in gray and samples with asterisks next to the sample number were not available for analysis in the Trofile assay or did not yield reportable data. ARV, antiretroviral therapy.

**B**

Consensus C	CTRPNNHTRKRSIRI..GPGQTFYATGDIIGDIRQANC	Consensus C	CTRPNNHTRKRSIRI..GPGQTFYATGDIIGDIRQANC
Consensus (Failures)	.....GPGQ.....	Consensus (Naïves)	.....GPGQ.....
007F*	-V-----T-----GPGQ-----N-----G-----E-----	001N	-----G-----GPGQ-----N-----N-----
008P	---G-----R-V-----GPGQ-----	003N	-I-G-----V-----GPGQ-----N-----K-----
009P	-S-G-Q-ERR-STR-I..GPGQ-QI-GRS-RT--GK-Y-	004N	-----Q--GF--GPGQA-----
011P	-I-G-----V-----GPGQ-----N-----K-----	005N	-I-G-----V-----GPGQ-----
012P*	-----R-M--GIGRQ-Q--M-R-----	008N	-----G-----V-----GPGQ-----N-----T-----
013P*	---GDRRRI--GPGQA-N-RDN-----K-Y-	010N	-----I-K-QR-----GPRAV-I-K-----K-----
017P	---G-----R--V--GPGQSI--NR-----K-Y-	013N	---S-----VG--GPGQ-I-----
018P	-I-G-----V-----GPGQ-----E-----V-----	016N	-----V-----V-----GPGQA-----G-----Y-----
019P*	-L-G-----R-----GPGQ-----A-----	017N	---VG-----V-----GPGQA-----N-----
020P	-----ITR-ISI..GPRP--TKNIGRDIK--Y-	018N	-I-G-----V-----GPGQA-----H-E-N-----
021P	---G-----R--V--GPGQSI--NR-----K-Y-	019N	-----G-----V-----GPGQ-----N-----
022P	-----R-R-M--GPGQV-----A-----N-----Y-----	020N	---G-I-----V-----GPGQA-PT-----Y-----
23P*	---G-----R-----GPGQ-----K-----	021N	-----G-----V-----GPGQA-----E-----
024P*	---G-----R-G--GPGQ-F-RT--N-----	024N	-----V-L--GPGQ-----E-----
028P*	-----K-R-R-K--GPRP-VTNH--N-----Y-----	027N	-----TR-TR--GPGQHAPPY-NTV--K-Y-
031P	---G-----Q-----GPGQ-----K-----	028N	-I--SH--QEGVRI.GPRA-VR-K-----K-Y-
033P	-----V-----GPGQ-----N-----Y-----	029N	---G--T-----GPGQ-----AVT-----
034P*	-----R-----GPR--PT-----	030N	---G-----V-----GPGQ-----N-----
037P	---G-I-R-G--GPGQA-R-SG--N-----Q-----	034N	---G-----V-----GPGQ-L-TN-----K-Y-
049P	-----Q-----GPGQ-----R-----	035N	-----G-----V-----GPGQ-----

Our results show that in a South African setting where patients are receiving antiretroviral therapy according to national and World Health Organization guidelines, 95% of patients failing therapy had at least one drug resistance mutation. The most common NRTI mutation was M184V/I in 87% of patients, with V106M/A (51%) and K103N (40%) the most common NNRTI resistance mutations. These patterns are consistent with data from previous HIV-1C studies.<sup>10,38-40</sup> However, we also found a high prevalence (13.3%) of the ETR resistance-associated M230L mutation, which occurs relatively rarely in NNRTI-failing patients. This finding may require follow-up studies. Data from this study also revealed

that 55% of patients failing therapy had TAMs compared with 32% of patients studied from the same city in 2005 to 2006.<sup>10</sup> Recent studies from Botswana, Malawi, and South Africa have also reported similar high proportions of TAMs in persons failing therapy under public sector antiretroviral programs with an apparent bias toward the TAM2 pathway in these Subtype C studies.<sup>33,41,42</sup> In contrast, a recent Subtype B study showed that 65% of virologically failing subjects harbored TAM1 pathway mutations<sup>8</sup> suggesting that there may be subtype-specific differences in TAM pathways. Other reasons for TAM pathway mutation differences may include differences in antiretroviral regimens, nadir CD4+ T-cell count before HAART

**TABLE 2.** V3 Loop-Based Methods for Coreceptor Use Prediction

Method	Percent of Sequences* Correctly Predicted	Percent of R5 Sequences Correctly Predicted	Percent of X4/D/M Sequences Correctly Predicted
11/25	78	90	55
Overall net V3 charge	75	71	81
C-PSSM	81	85	72
Geno2Pheno	84	86	82
Combined algorithm†	87	90	80

\*R5 refers to viruses using CCR5 only; X4/D/M refers to CXCR4-using viruses (CXCR4-only, dual tropic, and mixed populations).

†In the combined algorithm, concordant results from at least three of four methods (ie, the amino acids at positions 11 and/or 25, the overall net V3 charge, C-PSSM prediction, and Geno2Pheno prediction) were used.

commencement, duration of treatment before failure, and duration on a failing regimen. Overall, we found that more than 90% of patients failing therapy had at least one drug resistance mutation and 55% of patients harbored TAMs. To minimize the loss of future treatment options, improved adherence support mechanisms and better monitoring algorithms for patients on HAART in resource-poor settings will be required.

We also investigated here whether HAART-failing HIV-1C-infected patients had a higher proportion of X4/dual/mixed viruses compared with HAART-naïve patients. Although we found this to be the case, the patients failing HAART had lower median (nadir) CD4+ counts compared with HAART-naïve patients, and individuals possessing X4/dual/mixed viruses had significantly lower CD4+ T cell counts compared with those with R5-only viruses in both the HAART-naïve and HAART-failing arms of this study. These data therefore suggest that low CD4+ T-cell counts (and by extension the length of infection) rather than HAART is the possible main cause of X4/dual/mixed viruses, consistent with data from HIV-1 Subtype B studies.<sup>12,41</sup> However, only a longitudinal study can decisively determine whether there is higher proportion of emergence of X4/dual/mixed viruses in treated versus HAART-naïve patients with similar CD4+ T-cell counts. Nevertheless, our study underlines the importance of introducing CCR5 inhibitors relatively early in the course of HIV-1C infection for possible maximum benefit and to preserve other drugs for salvage use. Clinical trials are needed to determine the equivalence or superiority of CCR5 inhibitors as part of first-line or early regimens rather than as salvage therapy in HIV-1C settings.

Finally, the availability of virus phenotype and genotype data allowed us to assess the use of V3 loop sequenced-based methods for predicting viral tropism. Envelope sequence-based genotypic coreceptor prediction algorithms offer a simpler and less expensive means of analyzing viral tropism in patients and could facilitate easier and less expensive determination of whether a patient can be treated with CCR5 antagonists. Our data show that although genotypic methods are reliable for a majority of cases, they failed to correctly predict CXCR4 tropism in a significant minority of cases consistent with previous studies.<sup>43</sup> Although the Geno2Pheno and V3 charge methods achieved more than 80% sensitivity in

predicting CXCR4/dual tropism for this data set, there remains an urgent need to further investigate and develop better predictive algorithms, perhaps taking into account sequences outside of the V3 and more detailed analysis of V3 loop sequences using newer technologies able to better characterize V3 loop quasispecies diversity.

In summary, our observations confirm and extend the body of knowledge in examining coreceptor tropism directly in patients failing currently recommended regimens and comparing this with ARV-naïve patients in a HIV-1C setting. The presence of high proportions of patients with TAMs suggests that these mutations may be accumulating over time in this population as a result of inadequate viral suppression, perhaps as a consequence of poor immunologic and/or clinically driven monitoring in the absence of viral load testing. These results may suggest that in situations in which virologic monitoring is not possible, measures are needed to improve adherence and to develop better monitoring tools. Comparison of the prevalence of CXCR4-using viruses between ARV-naïve before initiating ART with the prevalence among treated patients revealed that there was a high prevalence of X4/dual/mixed viruses in patients failing treatment, possibly as a result of lower nadir CD4 counts in these patients, underlining the need to investigate the possible earlier use of CCR5 inhibitors before the development of X4/dual/mixed viruses. Our data also highlight the usefulness and limitations of genotypic coreceptor prediction methods in assessing whether HIV-1C-infected patients can be put on regimens that include CCR5 inhibitors. Longitudinal studies on viral coreceptor evolution in HIV-1C infections are warranted.

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**REFERENCES**

- UNAIDS. AIDS epidemic update. 2007. Available at: www.unaids.org. Accessed February 18, 2008.
- Taylor BS, Sobieszczyk ME, McCutchan FE, et al. The challenge of HIV-1 subtype diversity. *N Engl J Med.* 2008;358:1590–1602.
- South Africa Government online: the Government and Information System. Available at: http://www.info.gov.za/otherdocs/2003/aidsplan/report.pdf. Accessed February 25, 2008.
- UNAIDS Global Report. Factsheet - Sub-Saharan Africa. 2010. http://www.unaids.org/en/media/unaids/contentassets/documents/factsheet/2010/20101123\_FS\_SSA\_em\_en.pdf. Accessed May 11, 2011.
- Furtado MR, Callaway DS, Phair JP, et al. Persistence of HIV-1 transcription in peripheral-blood mononuclear cells in patients receiving potent antiretroviral therapy. *N Engl J Med.* 1999;340:1614–1622.
- Tobin NH, Learn GH, Holte SE, et al. Evidence that low-level viremia during effective highly active antiretroviral therapy result from two processes: expression of archival virus and replication of virus. *J Virol.* 2005;79:9625–9634.

7. WHO recommendations for clinical mentoring to support scale-up of HIV care, antiretroviral therapy and prevention in resource-constrained settings. *WHO Guidelines*. 2006. Available at: <http://www.who.int/hiv/pub/guidelines/clinicalmentoring.pdf>.
8. Cozzi-Lepri A, Phillips AN, Martinez-Picado J, et al. Rate of accumulation of thymidine analogue mutations in patients continuing to receive virologically failing regimens containing zidovudine or stavudine: implications for antiretroviral therapy programs in resource-limited settings. *J Infect Dis*. 2009;200:687–697.
9. Hosseinipour MC, van Oosterhout JGG, Weigel R, et al. The public health approach to identify antiretroviral therapy failure: high-level nucleoside reverse transcriptase inhibitor resistance among Malawians failing first-line antiretroviral therapy. *AIDS*. 2009;23:1127–1134.
10. Marconi VC, Sunpath H, Lu Z, et al. Prevalence of HIV-1 drug resistance after failure of a first highly active antiretroviral therapy regimen in KwaZulu Natal, South Africa. *Clin Infect Dis*. 2008;46:1589–1597.
11. Wallis C, Mellors JW, Venter F, et al. Varied patterns of HIV-1 drug resistance on failing first-line antiretroviral therapy in South Africa. *J Acquir Immune Defic Syndr*. 2010;53:480–484.
12. Hunt PW, Harrigan PR, Huang W, et al. Prevalence of CXCR4 tropism among antiretroviral-treated HIV-1-infected patients with detectable viremia. *J Inf Dis*. 2006;194:926–930.
13. Johnston ER, Zijenah LS, Mutetwa S, et al. High frequency of syncytium-inducing and CXCR4-tropic viruses among human immunodeficiency virus type 1 subtype C-infected patients receiving antiretroviral treatment. *J Virol*. 2003;77:7682–7688.
14. Connor RL, Sheridan KE, Ceradini D, et al. Change in coreceptor use correlates with disease progression in HIV-1-infected individuals. *J Exp Med*. 1997;185:621–628.
15. Scarlatti G, Tresoldi E, Bjornald A, et al. In vivo evolution of HIV-1 coreceptor usage and sensitivity to chemokine-mediated suppression. *Nat Med*. 1997;3:1259–1265.
16. Bjornald A, Sonnerborg A, Tscherning C, et al. Phenotypic characteristics of human immunodeficiency virus type 1 subtype C isolates of Ethiopian AIDS patients. *Aids Res Hum Retroviruses*. 1999;15:647–653.
17. Cecilia D, Kulkarni SS, Tripathy SP, et al. Absence of coreceptor switch with disease progression in human immunodeficiency virus infections in India. *Virology*. 2000;271:253–258.
18. Cilliers T, Nhlapo J, Coetzer M, et al. The CCR5 and CXCR4 coreceptors are both used by human immunodeficiency virus type 1 primary isolates from subtype C. *J Virol*. 2003;77:4449–4456.
19. Ndung'u T, Sepako E, McLane MF, et al. HIV-1 subtype C in vitro growth and coreceptor utilization. *Virology*. 2006;347:247–260.
20. Tscherning CA, Alaeus RF, Fredriksson A, et al. Differences in chemokine coreceptor usage between genetic subtypes of HIV-1. *Virology*. 1998;241:181–188.
21. Reeves JD, Coakley E, Petropoulos CJ, et al. An enhanced-sensitivity Trofile™ HIV coreceptor tropism assay for selecting patients for therapy with entry inhibitors targeting CCR5: a review of analytical and clinical studies. *J Viral Entry*. 2009;3:94–102.
22. Trinh L, Han D, Huang W, et al. Technical validation of an enhanced sensitivity Trofile™ HIV co-receptor tropism assay for selecting patients for therapy with entry inhibitors targeting CCR5. *Antiviral Ther*. 2008;13:128.
23. Coakley E, Petropoulos CJ, Whitcomb JM. Assessing chemokine co-receptor usage in HIV. *Curr Opin Infect Dis*. 2005;18:9–15.
24. Westby M, Lewis M, Whitcomb J, et al. Emergence of CXCR4-using human immunodeficiency virus type 1 (HIV-1) variants in a minority of HIV-1-infected patients following treatment with the CCR5 antagonist maraviroc is from a pretreatment CXCR4-using virus reservoir. *J Virol*. 2006;80:4909–4920.
25. Singh A, Page T, Moore PL, et al. Functional and genetic analysis of coreceptor usage by dualtropic HIV-1 subtype C isolates. *Virology*. 2009;393:56–67.
26. Tamura K, Dudley J, Nei M, Kumar S. MEGA4: molecular evolutionary genetics analysis (MEGA) software version 4.0. *Mol Biol Evol*. 2007;24:1596–1599.
27. Coetzer M, Cilliers T, Ping LH, et al. Genetic characteristics of the V3 region associated with CXCR4 usage in HIV-1 subtype C isolates. *Virology*. 2006;356:95–105.
28. Fouchier RA, Brouwer M, Broersen SM, et al. Simple determination of human immunodeficiency virus type 1 syncytium-inducing V3 genotype by PCR. *J Clin Microbiol*. 1995;33:906–911.
29. Fouchier RA, Groenink M, Kootstra NN, et al. Phenotype-associated sequence variation in the third variable domain of the human immunodeficiency virus type 1 gp 120 molecule. *J Virol*. 1992;66:3183–3187.
30. Kuiken CL, de Jong JJ, Baan E, et al. Evolution of the V3 envelope domain in proviral sequences and isolates of human immunodeficiency virus type 1 during transition of the viral biological phenotype. *J Virol*. 1992;66:5704.
31. Whitcomb J, Parkin N, Cheppey C, et al. Broad NRTI cross-resistance in HIV-1 clinical isolates. *J Infect Dis*. 2003;188:992–1000.
32. Stanford HIV Drug Resistance Database. Available at: <http://hibv.stanford.edu/>. Accessed May 11, 2009.
33. Novitsky V, Wester W, Degroot V, et al. The reverse transcriptase 67N 70R 215Y genotype is the predominant TAM pathway associated with virologic failure among HIV type 1C-infected adults treated with ZDV/ddI-containing HAART in Southern Africa. *AIDS Res Hum Retroviruses*. 2007;23:868–878.
34. Briggs DR, Tuttle DL, Sleasman JW, et al. Envelope V3 amino acid sequence predicts HIV-1 phenotype (co-receptor usage and tropism for macrophages). *AIDS*. 2000;14:2937–2939.
35. Cann AJ, Churcher MJ, Boyd M, et al. The region of the envelope gene of human immunodeficiency virus type 1 responsible for determination of cell tropism. *J Virol*. 1992;66:305–309.
36. Rizzuto C, Wyatt R, Hernandez-Ramos N, et al. A conserved HIV gp120 glycoprotein structure involved in chemokine receptor binding. *Science*. 1998;280:1949–1953.
37. Wu X, Parast AB, Richardson BA, et al. Neutralization escape variants of human immunodeficiency virus type 1 are transmitted from mother to infant. *J Virol*. 2006;80:835–844.
38. Brenner B, Oliveira M, Moisi D, et al. A V106M mutation in HIV-1 clade C viruses exposed to efavirenz confers cross resistance to nonnucleoside reverse transcriptase inhibitors. *AIDS*. 2003;17:F1–F5.
39. Kantor R, Katzenstein DA, Efron B, et al. Impact of HIV-1 subtype and antiretroviral therapy on protease and reverse transcriptase genotype: results of a global collaboration. *PLoS Med*. 2005;2:e112.
40. Loomba H, Brenner B, Parniak MA, et al. Genetic divergence of human immunodeficiency virus type 1 Ethiopian clade C reverse transcriptase (RT) and rapid development of resistance against nonnucleoside inhibitors of RT. *Antimicrob Agents Chemother*. 2002;46:2087–2094.
41. Briz V, Poveda E, del Mar Gonzalez M, et al. Impact of antiretroviral therapy on viral tropism in HIV-infected patients followed longitudinally for over 5 years. *J Antimicrob Chemother*. 2008;65:405–410.
42. Irlbeck DM, Amrine-Madsen H, Kitrinos KM, et al. Chemokine (C-C motif) receptor 5-using envelopes predominate in dual/mixed-tropic HIV from the plasma of drug-naive individuals. *AIDS*. 2008;22:1425–1431.
43. Low AJ, Dong W, Chan D, et al. Current V3 genotyping algorithms are inadequate for predicting X4 co-receptor usage in clinical isolates. *AIDS*. 2007;21:F17–F24.