

Impact of pre-existing drug resistance on risk of virological failure in South Africa

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Objectives: There is conflicting evidence on the impact of pre-existing HIV drug resistance mutations (DRMs) in patients infected with non-B subtype virus.

Methods: We performed a case-cohort substudy of the AIDS Drug Resistance Surveillance Study, which enrolled South African patients initiating first-line efavirenz/emtricitabine/tenofovir. Pre-ART DRMs were detected by Illumina sequencing of HIV *pol* and DRMs present at <20% of the viral population were labelled as minority variants (MVs). Weighted Cox proportional hazards models estimated the association between pre-ART DRMs and risk of virological failure (VF), defined as confirmed HIV-1 RNA ≥ 1000 copies/mL after ≥ 5 months of ART.

Results: The evaluable population included 178 participants from a randomly selected subcohort (16 with VF, 162 without VF) and 83 additional participants with VF. In the subcohort, 16% of participants harboured ≥ 1 majority DRM. The presence of any majority DRM was associated with a 3-fold greater risk of VF ($P=0.002$), which increased to 9.2-fold ($P<0.001$) in those with <2 active drugs. Thirteen percent of participants harboured MV DRMs in the absence of majority DRMs. Presence of MVs alone had no significant impact on the risk of VF. Inclusion of pre-ART MVs with majority DRMs improved the sensitivity but reduced the specificity of predicting VF.

Conclusions: In a South African cohort, the presence of majority DRMs increased the risk of VF, especially for participants receiving <2 active drugs. The detection of drug-resistant MVs alone did not predict an increased risk of VF, but their inclusion with majority DRMs affected the sensitivity/specificity of predicting VF.

Introduction

The WHO estimates that more than 38 million people are living with HIV, nearly two-thirds of whom live in sub-Saharan Africa. In 2014, UNAIDS introduced the 90-90-90 targets for HIV control, including that 90% of those on ART are virologically suppressed. To reach these treatment goals, additional progress will need to be made in sub-Saharan Africa, where rates of first-line ART failure remain high^{1,2} and increasing rates of ART resistance have been reported.³ While pre-ART HIV drug resistance testing is routine in the USA and Europe, it is generally unavailable in sub-Saharan Africa due to its cost.

In addition, there is controversy over the impact of pre-treatment genotypic resistance testing in sub-Saharan Africa due to the conflicting evidence regarding the association of

pre-existing HIV drug resistance mutations (DRMs) and virological failure (VF) in patients infected with non-B subtype virus. It has been well documented in US and European studies that the presence of HIV DRMs predicts increased risk of VF.⁴⁻⁶ While there have been similar findings from Africa for patients on older ART regimens,⁷⁻⁹ a recent analysis of the ANRS Treatment-as-Prevention (TasP) trial found no association between pre-existing resistance mutations to NNRTIs and rates of virological suppression for participants receiving an efavirenz/emtricitabine/tenofovir (EFV/FTC/TDF) regimen.¹⁰

This controversy extends to the clinical impact of drug-resistant minority variants (MVs).¹¹ While traditional Sanger-based genotypic tests for HIV-1 drug resistance employ techniques that detect resistance-associated mutations present in at least 20% of

the viral population,^{12,13} these assays fail to detect the presence of low-frequency drug-resistant MVs within the population of HIV-1 quasispecies in an infected individual. Next-generation sequencing techniques can detect such MVs and the increasingly higher throughput and decreasing costs of these platforms suggest that it may have an eventual role in increasing access to HIV drug resistance testing in low- and middle-income countries. In US and European studies, the presence of pre-treatment NNRTI-resistant MVs is associated with more than twice the risk of VF, even after controlling for medication adherence and other potential confounders.¹⁴ However, studies performed in sub-Saharan Africa have yielded mixed results,^{15–17} contributing to the confusion surrounding the impact of HIV resistance mutations on VF in patients infected with non-B subtype virus.

In this study, we performed a case-cohort analysis of the KwaZulu-Natal (KZN) HIV AIDS Drug Resistance Surveillance Study (ADReSS), which enrolled treatment-naïve persons with HIV initiating ART at peri-urban and rural clinics in South Africa.¹⁸ Using next-generation sequencing, we assessed the impact of both majority and minority HIV DRMs on the risk of VF in those initiating a first-line NNRTI-based ART regimen.

Methods

Study population and design

We performed a case-cohort substudy of ADReSS, which enrolled 1000 patients initiating first-line EFV/FTC/TDF in KZN, South Africa.¹⁹ Participants were equally enrolled between July 2014 and July 2018 from two centres: a peri-urban hospital in Chatsworth [RK Kahn Hospital (RKK)] and a rural hospital in Bethesda [Bethesda Hospital (BTD)]. In this case-cohort study design, cases were defined as participants with VF based on confirmed (at least 2) plasma HIV-1 RNA ≥ 1000 copies/mL after ≥ 5 months of ART. All cases and a random subcohort of individuals (equally divided between the two enrolling centres) were selected for drug resistance testing at the pre-ART timepoint. Participants were excluded from the analysis if they were not receiving an NNRTI-based ART regimen, were lost to follow-up or did not have available pre-ART plasma samples.

PCR amplification and control library construction

Viral RNA extraction was performed on 1 mL of plasma using the Nuclisens miniMAG (bioMérieux). Synthesis of cDNA was performed using an integrase-specific primer and the Superscript IV reverse transcriptase. The HIV-1 *pol* gene (HXB2 nucleotides 2040–3810 encompassing protease amino acids 1–99 and reverse transcriptase amino acids 1–420) was PCR amplified using Platinum Taq HiFi and a conserved, nested primer set (first round forward primer: 5'-TGGAAATGTGGAAAGGACACCAAAT-3', reverse primer: 5'-CTAGGGGAGGGGTATTAAACAACTC-3'; second round forward primer: 5'-AAAGGAAGGACACCAAATGAAAGA-3', reverse primer: 5'-GAGGGGTATTAAACAACTCCA-3'). PCR purification was performed using Agencourt AMPure XP beads. PCR amplicons were sheared (Covaris) and Illumina barcoded libraries were constructed and pooled. Sequencing was performed on the Illumina MiSeq platform with an average of 3000–4000 \times coverage.

The accuracy of the deep sequencing platforms was evaluated with a control library of clonal HIV sequences mixed at known concentrations. The PCR amplicons from cloned sequences were gel purified and quantified by Nanodrop spectrophotometry. A control library was created by mixing the clones at concentrations of 68.4%, 25%, 5%, 1%, 0.5% and 0.1%. The frequency of false-positive MV calls was assessed by the presence of MVs at all control library nucleotide positions that should be invariant (i.e. all amplicons have the same base) and the upper range of the assay

error rate quantified as 3 SDs above the mean MV percentage detected amongst the invariant bases.

Sequence analysis and detection of HIV drug resistance

Pre-ART DRMs were detected by multiplexed Illumina sequencing of HIV *pol* and sequence analysis performed using PAsSeq software.^{17,20} Personalized genotypic susceptibility scores (GSSs) at varying MV thresholds (0.5%–20%) were calculated using the Stanford HIV database. The Stanford HIV database provides a weighted penalty score for the effect of every resistance mutation and antiretroviral (ARV) medication, with a score of 0 if there is no expected effect to 60 for high-level resistance. For each sequence, the estimated level of resistance for each ARV will be determined by adding all of the penalty scores for each of the DRMs present. The GSS of each ARV was defined as the following: 1 (Stanford penalty score 0–9), 0.75 (Stanford penalty score 10–14), 0.5 (Stanford penalty score 15–29), 0.25 (Stanford penalty score 30–59) and 0 (Stanford penalty score ≥ 60). The GSS of the ART regimen was the sum of the GSSs for each ARV. DRMs present at $\geq 20\%$ of the viral population were labelled as 'majority' variants likely to be detectable by Sanger sequencing.

Statistical analysis

The analysis used a weighted stratified Cox proportional hazards model incorporating both the random subcohort participants and the non-subcohort cases, with weights determined by the inverse of the sampling fraction or case designation. Stratification was done by site. This model estimated an HR as the quantification of the association of HIV resistance and risk of VF. A comparison of GSS distributions for the subcohort participants by the use of 'majority only' versus 'majority and all minority' DRMs was performed by χ^2 testing.

Ethics

Written informed consent was provided by all study participants for the use of stored samples in HIV-related research. This study was approved by the University of KZN Biomedical Research Ethics Committee, Partners and Emory University Institutional Review Boards.

Results

Participant characteristics

The evaluable case-cohort sample included 178 participants from the randomly selected subcohort (16 with VF and 162 without VF) and 83 additional participants with VF (non-subcohort cases). Within the subcohort, the mean age was 35 years and 64% of participants were female (Table 1). Between the subcohort and additional cases, 97% of participants received EFV/FTC/TDF. Participants were almost evenly divided between those enrolled in the peri-urban and rural sites. All participants harboured clade C HIV.

Presence of HIV majority drug resistance and impact on treatment failure

In the random subcohort, 16% of participants harboured at least one majority DRM (present at $\geq 20\%$ of the viral population) that conferred intermediate or greater ART resistance (Stanford score ≥ 30 , Figure 1). The GSSs were calculated using these majority resistance mutations: 81% of participants in the subcohort had a GSS of 3, 15% had a GSS of 2–2.75 and 4% of participants had a GSS of < 2 , corresponding to < 2 fully active ARVs (Figure 2). The presence

Table 1. Baseline characteristics of parent and subcohort population

Characteristic	Non-cohort case (N = 83)	Random subcohort		
		case (N = 16)	non-case (N = 162)	total (N = 178)
Enrolment site, n (%)				
RKK	43 (52)	5 (31)	85 (52)	90 (51)
BTD	40 (48)	11 (69)	77 (48)	88 (49)
Age at enrolment, years				
mean (SD)	31.33 (8.67)	33.94 (12.30)	35.12 (10.84)	35.02 (10.95)
median (IQR)	30 (24–38)	29.50 (23.50–39.00)	32 (27–42)	32 (27–42)
min–max	18–53	21–61	20–66	20–66
Sex, n (%)				
female	45 (54)	10 (6)	104 (64)	114 (64)
male	38 (46)	6 (38)	58 (36)	64 (36)
ARV regimen, n (%)				
EFV/FTC/TDF	78 (94)	15 (94)	161 (99)	176 (99)
EFV/3TC/ABC	1 (1)	1 (6)	1 (1)	2 (1)
EFV/3TC/ZDV	1 (1)	0 (0)	0 (0)	0 (0)
NVP/FTC/TDF	2 (2)	0 (0)	0 (0)	0 (0)
NVP/3TC/TDF	1 (1)	0 (0)	0 (0)	0 (0)

EFV, efavirenz; FTC, emtricitabine; TDF, tenofovir disoproxil fumarate; 3TC, lamivudine; ABC, abacavir; ZDV, zidovudine; NVP, nevirapine.

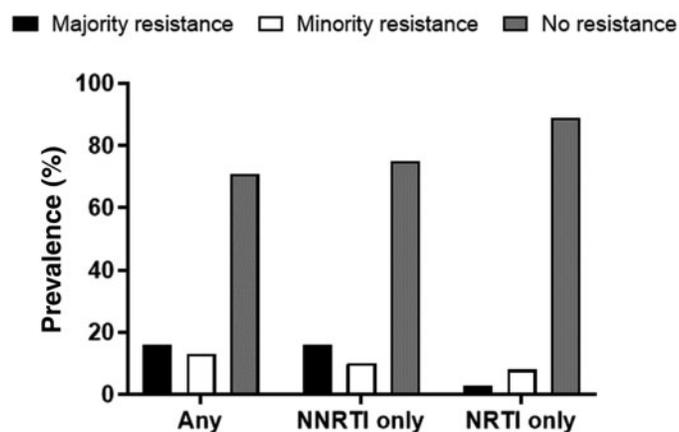


Figure 1. Prevalence of HIV DRMs by ARV class and frequency. Prevalence calculations performed amongst the subcohort population. Percentage of individuals harbouring minority resistance mutations excludes those with majority resistance. Majority resistance mutations refer to mutations conferring intermediate or greater resistance to the participant's ARV regimen and present at $\geq 20\%$ of the viral population. Minority resistance mutations refer to mutations present at $\geq 0.5\%$ and $< 20\%$ of the viral population.

of any significant majority DRM was associated with a 3-fold risk of VF [HR (95% CI) = 3.0 (1.5–5.9); $P = 0.002$]. In those with < 2 active drugs due to majority DRMs, the risk of VF increased to 9-fold [HR (95% CI) = 9.2 (3.3–26.0); $P < 0.001$] compared with those with 3 active drugs. The most prevalent resistance mutations in the subcohort were K103N (9% of participants), E138A (8%), V106M (4%) and M184V (3%) (Table S1, available as [Supplementary data](#) at JAC Online). Amongst those who experienced eventual VF, the

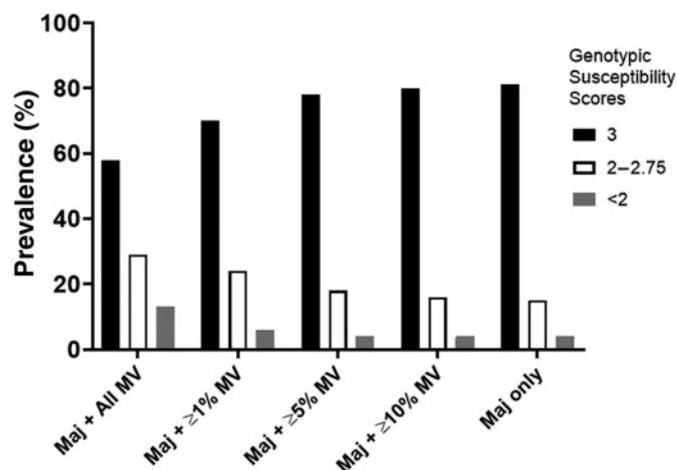


Figure 2. Proportion of subcohort participants at each GSS level calculated with majority DRMs alone (Maj only) or in the presence of varying frequencies of minority DRMs. Maj, majority DRMs; MV, minority DRMs present at the specified frequency.

most common pre-treatment majority resistance mutations were K103N (24% of VF participants), M184V (17%), V106M (10%), E138A (6%), P225H (6%) and K65R (6%).

Effect of HIV minority DRMs

Based on the control library, the upper range for the assay error was approximately 0.2% and we conservatively used 0.5% as the MV assay limit of detection. Thirteen percent of participants in the random subcohort harboured any minority DRMs in the absence of

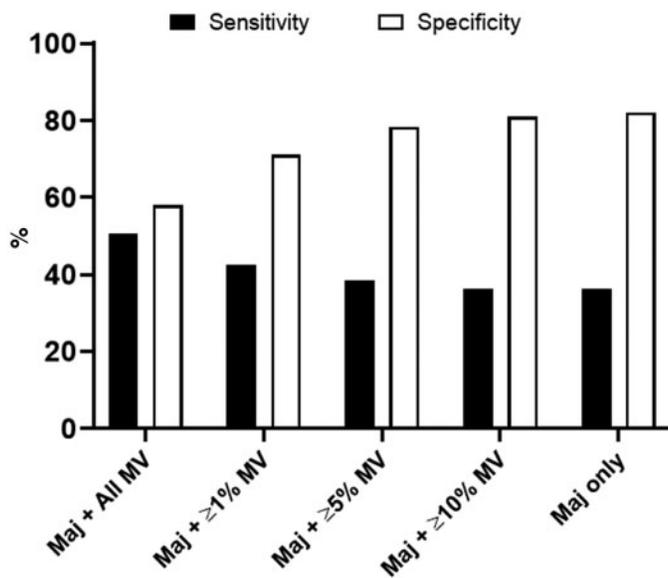


Figure 3. Sensitivity and specificity of predicting VF based on the presence of at least one majority and/or minority DRMs. Maj, majority DRMs; MV, minority DRMs present at the specified frequency.

majority resistance (Figure 1). The GSSs were calculated using majority resistance mutations and with minority resistance mutations at varying threshold frequencies (Figure 2). Incorporating minority DRMs significantly decreased the proportion of participants who were receiving three fully active drugs and increased those with GSS < 3 ('majority only' versus 'majority and all MVs', GSS = 3: 81% versus 58% of subcohort participants; GSS of 2–2.75: 15% versus 29%; and GSS < 2: 4% versus 13%, $\chi^2 P < 0.001$). The most commonly detected high-level majority DRMs (e.g. K103N, V106M and M184V) were less commonly detected as MVs (Table S1, Figure S1). Among those with VF, the most common pre-treatment minority resistance mutations included E138K (7% of VF participants), K103N (6%), D67N (4%) and F227L (4%). Presence of MVs without majority DRMs had little impact on the risk of VF (risk of VF for MVs alone: HR 0.79, $P = 0.57$). Inclusion of pre-ART MVs with majority DRMs improved the sensitivity but reduced the specificity of predicting VF of first-line ART (Figure 3).

Discussion

South Africa has the largest ART programme worldwide and optimizing ART success has important implications for the eventual control of the epidemic. However, there remains controversy over the utility of pre-treatment drug resistance testing, including the impact of low-frequency drug-resistant MVs. In this study, we evaluated the impact of pre-existing majority and minority DRMs in a cohort of participants starting first-line NNRTI-based ART enrolled in both rural and peri-urban centres in South Africa. The results demonstrate that the presence of majority DRMs increased the risk of VF, an effect largely driven by the presence of dual-class resistance. The detection of drug-resistant MVs alone did not predict a significant increased risk of VF compared with majority DRMs, but their inclusion with majority DRMs improved the sensitivity but decreased the specificity of predicting VF.

While Sanger sequencing-based HIV resistance genotyping remains an integral part of HIV clinical care in the USA and Europe, it remains inaccessible to substantial proportions of patients in low- and middle-income countries. Furthermore, there is controversy over the impact of pre-existing DRMs in these populations who are infected with non-B subtype HIV.^{7–10} Our results provide further evidence supporting the role of pre-treatment drug resistance testing as single DRMs generally detectable by Sanger sequencing ($\geq 20\%$ of the viral population) were associated with a 3-fold increase in VF and <2 fully active ARVs conferred a 9-fold increase in the risk of VF. The finding that even a single DRM was associated with a significantly increased risk of VF is in direct contrast to the recently published analysis of the ANRS TasP trial. That study confirmed the negative impact of dual-class ART resistance, but reported that single-class resistance mutations were not associated with increased rates of VF in those initiating the same ART regimen (EFV/FTC/TDF) as our trial.¹⁰ The reason for differences in outcomes between our studies remains unclear as both used Illumina deep sequencing and the 20% threshold for approximating viral resistance mutations that would be detectable by Sanger sequencing. Additional studies are needed to further explore these findings, including the substantial impact that suboptimal ART adherence may have on VF rates.²¹

Due to the complexity of the HIV quasispecies, low-frequency minority DRMs can be present at <20% of the viral population, which is generally considered the limit of detection for conventional Sanger-based genotyping assays. The presence of these MVs has been shown to increase the risk of VF in US and European populations. In a pooled analysis of 10 studies of treatment-naïve participants initiating an NNRTI-based regimen, the presence of a baseline minority HIV DRM was associated with more than twice the risk of VF.¹⁴ However, the clinical impact of HIV MVs on VF in African patients remains controversial as our results are consistent with other studies showing the limited impact of MVs, especially those present at the lowest frequencies.^{16,17} One potential explanation for the differences is that the US and European studies detected a relatively large number of NNRTI-resistant MVs that confer high-level resistance (e.g. K103N and Y181C).¹⁴ In contrast, relatively few participants in our study were found to harbour major NNRTI resistance mutations as MVs. Whether this is potentially due to lower levels of transmitted drug resistance or HIV subtype-specific differences in *de novo* resistance emergence is unclear but highlights the importance that future studies assess both the type of minority resistance mutations present and their frequency.

An analysis of the A5208/OCTANE Trial 1 study using next-generation sequencing with unique molecular identifiers reported that linked, dual-class resistance mutations increased the risk of treatment failure.²² One limitation of our study was the inability to assess resistance linkage and additional studies are needed to explore underlying differences in the frequency of dual-class resistance between US/European and African populations. An additional limitation of this study is its focus on patients initiating first-line NNRTI-based ART. While integrase strand-transfer inhibitor (INSTI)-based ART regimens have increasingly become available in resource-limited settings, there remains concern about their use for all populations.²³ Optimal monitoring and use of INSTIs in Africa remains to be determined given concerns about the existing

spectrum of drug resistance in Africa^{24,25} and reports of dolutegravir treatment failure in non-B subtype-infected patients.^{26–28}

The scale-up of HIV drug resistance testing in low- and middle-income countries remains both a challenge and an area of increasing need. As next-generation sequencing-based technologies mature and decrease in cost, it is hoped that these platforms may play an important role in increasing access to HIV genotyping worldwide.^{29,30} In this study, we used next-generation sequencing to show that HIV DRMs at $\geq 20\%$ of the viral population were associated with significantly increased risk of VF in South African patients initiating first-line NNRTI-based ART. The detection of drug-resistant MVs alone did not significantly increase the risk of VF, but their inclusion with majority resistance mutations affected the sensitivity and specificity of predicting VF depending on the threshold of detection. Additional studies are needed to define the optimal MV detection limit and to explore the differential impact of minority DRMs in the US and European versus African populations.

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Transparency declarations

J.Z.L. has served as a consultant for Jan Biotech and AbbVie. R.P. has research funding or consulting honoraria from MSD, Gilead and ViiV. V.C.M. received investigator-initiated research grants and consultation fees from Eli Lilly, Bayer, Gilead Sciences and ViiV. D.R.K. has received research funding or consulting honoraria from AbbVie, Gilead, GlaxoSmithKline, Janssen, Merck and ViiV. All other authors: none to declare.

J.Z.L., H.R., V.C.M. and D.R.K. were involved in the design of the study. M.-Y.M., J.B., S.P., H.S. and V.C.M. were involved in the collection of samples. J.Z.L., N.S., M.C.C., A.J., K.R., H.R., M.N.-J., R.P., B.J., A.E., V.C.M. and D.R.K. were involved in the generation and analysis of the data. All authors were involved in the preparation and editing of the manuscript.

Supplementary data

Table S1 and Figure S1 are available as [Supplementary data](#) at JAC Online.

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