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Emergence of Human Immunodeficiency Virus-1 Drug Resistance During the 3-Month World Health Organization-Recommended Enhanced Adherence Counseling Period in the CART-1 Cohort Study

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Background. In resource-limited settings, the World Health Organization recommends enhanced adherence counseling (EAC) for individuals with an unsuppressed human immunodeficiency virus (HIV)-1 viral load (VL) and to remeasure VL after 3 months to avoid unnecessary regimen switches. In cases in which this follow-up VL remains unsuppressed, a regimen switch is indicated. We aimed to assess levels of HIV-1 drug resistance before and after the EAC period among people with ongoing viremia (≥80 c/mL) after EAC.

Methods. We included adult participants of the CART-1 cohort study conducted in Lesotho who had a VL ≥80 c/mL after EAC. Paired plasma samples (before and after EAC) were analyzed by next-generation sequencing. We assessed the prevalence of resistance-associated mutations and viral susceptibility scores to each participant's antiretroviral therapy (ART) regimen (range, 0–3; 3 indicates complete susceptibility).

Results. Among 93 participants taking nonnucleoside reverse-transcriptase inhibitor-based ART with an initial VL ≥1000 copies/mL who received a follow-up VL test after EAC, 76 still had a VL ≥80 copies/mL after EAC, and paired samples were available for 57 of 76. The number of individuals without full susceptibility to any drug in their regimen increased from 31 of 57 (54.4%) before to 36 of 57 (63.2%) after EAC. Median susceptibility scores dropped from 0.5 (interquartile range [IQR] = 0.25–) to 0.25 (IQR = 0.25–1) during the EAC period ($P = .16$).

Conclusions. Despite high levels of resistance before EAC, we observed a slight decline in susceptibility scores after EAC. The risk of further accumulation of resistance during EAC has to be balanced against the benefit of avoiding unnecessary switches in those with spontaneous resuppression after EAC.

Keywords. drug resistance; genotypic resistance testing; HIV; sub-Saharan Africa.

Sub-Saharan Africa (SSA) is home to 25.6 million people with human immunodeficiency virus (HIV)-1 (PWH), corresponding to 68% of the global burden of the epidemic [1]. The number of PWH who receive antiretroviral therapy (ART) rose rapidly across SSA to an estimated 16.4 million by the end of 2018 [1–3]. However, the emergence of HIV drug resistance (HIVDR) is a key threat to the achievements made by improving access to ART in SSA and endangers the success of the universal test-and-treat strategy [4, 5]. Projections show that ~890 000 acquired immune deficiency syndrome (AIDS) deaths and costs of US ~$6.5 billion attributable to HIVDR are expected in SSA between 2016 and 2030 [6]. Currently, for PWH with a viral load (VL) ≥1000 copies/mL (c/mL) while on first-line ART in resource-limited settings, the World Health Organization (WHO) recommends adherence counseling with a follow-up VL test within 3 to 6 months [7]. According to guidelines in Lesotho, nurse-administered enhanced adherence counseling (EAC) sessions should begin as soon as possible after treatment failure and focus on reviewing a patient's adherence, identifying potential barriers to good adherence, and developing and implementing an adherence plan. Only a sustained VL ≥1000 c/mL after EAC triggers a switch to second-line ART, generally consisting of ritonavir-boosted lopinavir

plus 2 nucleoside reverse-transcriptase inhibitors (NRTIs) at the time of this study.

The rationale for this approach is to avoid unnecessary regimen switches for PWH where ongoing poor adherence rather than drug resistance is the cause of unsuppressed VL, notably in settings where drug resistance testing or drug level testing are unavailable. Several cohort studies from resource-limited settings endorse this approach, reporting up to 50% resuppression after EAC [8]. This may both save additional costs of second-line treatment and avoid further complications on second-line regimens, such as a higher pill burden or side effects. Likewise, in patients who do require switching to second-line ART due to ongoing viremia despite EAC, increased treatment literacy after EAC might help prevent the emergence of drug resistance on the new ART regimen.

However, the risk of the current EAC approach is that PWH with drug resistance, who qualify for switch to second-line ART and who are already taking their medication correctly, are delayed from receiving appropriate care. In these patients, ongoing viral replication may drive the accumulation of further HIVDR, thereby limiting future treatment options. Furthermore, many ART programs subsequently fail to switch PWH with virologic failure promptly [9], potentially leading to morbidity, mortality, and onward transmission of HIV [10–14].

The CART-1 study (NCT02126696) assessed virologic outcomes among patients attending routine ART care at 10 clinics in Lesotho [15, 16]. This substudy includes participants who had an unsuppressed VL, received EAC, and then had a second VL. Comparing resistance-associated mutations (RAMs) detectable through next-generation sequencing (NGS) before and after EAC, we assess to what extent the "waiting period" during EAC contributed to an accumulation of further RAMs, with the hypothesis that further RAMs might emerge during this period.

METHODS

Patient Consent Statement

The "Comorbidities and Virologic Outcome among Patients on Antiretroviral Therapy in Rural Lesotho" (CART-1, NCT02126696) [15] study was approved by the National Health Research Ethics Committee of the Ministry of Health of Lesotho (ID 01-2014) and the "Ethikkommission Nordwest-und Zentralschweiz" (EKNZ) in Switzerland (ID 2014-029). Participants provided written informed consent.

Participants and Sample Collection

Samples were obtained from the CART-1 study conducted in 2014. CART-1 involved a cross-sectional assessment of routine VL among patients on first-line ART attending care at 10 facilities in 2 districts of Lesotho, followed by a cohort study involving patients with a VL ≥80 c/mL. Clinical and virological outcomes of this cohort have been published [15]. Among 110

adult participants with an initial VL ≥1000 c/mL, 3 (2.7%) died, 5 (4.6%) switched to second-line ART after their first VL, and 9 (8.2%) were lost to follow-up. Of the remaining 93, 17 (18.3%), 12 (12.9%), and 64 (68.8%) had a follow-up VL of <80, 80–999, and ≥1000 c/mL, respectively. We were able to obtain and analyze plasma samples, which had been stored at −80°C, for 10 of 12 and 47 of 64 participants with a follow-up VL of 80–999 c/mL (FUVL80-999 group) and ≥1000 c/mL (FUVL ≥1000 group), respectively.

Sample Preparation and Sequencing

We used Ilumina MiSeq NGS (Illumina, San Diego, CA) to analyze the viral protease and reverse-transcriptase regions in paired plasma samples. Ribonucleic acid was extracted from up to 1 mL blood plasma using the Maxwell RSC Viral Total Nucleid Acid Purification Kit (Promega Corporation, Madison, WI). All subsequent laboratory procedures were performed according to a validated protocol, which has been described elsewhere [17], with the slight modification that only 1 complementary deoxyribonucleic acid fragment was synthesised (primer 3' UNI-KS-A/G-4) as a template for amplification of both the protease and the reverse-transcriptase regions.

Analysis of Drug-Susceptibility Scores Before and After Enhanced Adherence Counseling

Consensus sequences for each sample were obtained by Minvar 2.2 using the nucleotide present at maximum frequency at each position [18]. Using these consensus sequences, we obtained drug resistance scores to each drug in a patient's 3-drug regimen from the Stanford HIV Drug Resistance Database [19, 20]. Based on the resistance scores, we calculated drug-specific susceptibility scores. High-level resistance, intermediate resistance, low-level resistance, potential low-level resistance, and susceptible were assigned values of 0.00, 0.25, 0.50, 0.75, and 1.00, respectively, as has been described elsewhere [21]. Values for each drug of a participant's 3-drug regimen (possible range: 0.00–1.00) were added to obtain their overall regimen susceptibility score (possible range: 0.00–3.00), with lower scores indicating lower susceptibility (higher resistance). We used a sign test to compare regimen susceptibility scores before and after EAC in the full dataset as well as stratified by VL after EAC, ie, in the FUVL80-999 and the FUVL ≥1000 group.

Analysis of Frequency of Viral Drug Resistance Before and After Enhanced Adherence Counseling

We determined the presence of RAMs and their patient-level variant frequencies using Minvar 2.2 [18]. We considered changes in frequencies of major RAMs, according to IAS-USA 2019 edition [22], at all genomic positions in which major RAMs were observed with a frequency of >5%, the lowest threshold recommended by Huber et al [18] for analyses using MinVar. If different major RAMs, according to Wensing et al [22], were present at a given position, we considered the sum of their

frequencies. This analysis was carried out for the full dataset as well as separately for the FUVL ≥1000 and the FUVL80-999 group. We used paired *t* tests to compare these variant frequencies before and after EAC.

RESULTS

Study Population

Table 1 displays characteristics of the FUVL ≥1000 and FUVL80-999 populations. All participants were on an ART regimen consisting of 1 non-NRTI (NNRTI), either efavirenz or nevirapine, and a NRTI backbone, consisting of lamivudine with either zidovudine or tenofovir disoproxil fumarate. During EAC, the median VL in the FUVL ≥1000 and FUVL80-999 groups changed from a median of 13 971 c/mL and 3515 c/mL to 9738 c/mL and 476 c/mL, respectively.

Prevalence of Resistance-Associated Mutations

Among the participants studied here, ie, individuals whose VL did not suppress to <80 c/mL after EAC, 53 of 57 (93.0%) harbored at least 1 major NNRTI RAM and 50 of 57 (87.7%) harbored at least 1 major NRTI RAM already before EAC (Figure 1). Stratified by subsequent VL, 43 of 47 (91.5%) in the FUVL ≥1000 group and 10 of 10 (100.0%) in the FUVL80-999 had 1 major RAM before EAC, and 33 of 47 (70.2%) and 6 of

10 (60.0%), respectively, had 4 or more (Supplementary Figure S1). Although the number of participants without a single major RAM remained unchanged after EAC (Figure S1), 16 of 57 (28.1%) participants accumulated major RAMs in genomic positions that had not harbored RAMs before EAC. The total number of major RAMs detected throughout the study population increased from 278 (231 in the FUVL ≥1000 group and 47 in the FUVL80-999 group) before EAC to 287 (241 and 46) after EAC. The total number of RAMs (major and accessory) increased from 405 (343 in the FUVL ≥1000 group and 62 in the FUVL80-999 group) to 425 (363 and 62) (Supplementary Table S1).

Change of Susceptibility Score to Current Antiretroviral Therapy Regimen During Enhanced Adherence Counseling

Thirty-one of 57 (54.4%) were not fully susceptible to any drug in their regimen already before EAC, which increased to 36 of 57 individuals (63.2%) after EAC. After EAC, susceptibility scores (1) remained unchanged for 49 participants, (2) increased for 2 participants, and (3) decreased for 6 participants. Median susceptibility scores decreased from a 0.5 (interquartile range [IQR] = 0.25–1) before to 0.25 (IQR = 0.25–1, *P* = .16) after EAC (Figure 2). In the FUVL ≥1000 group, median regimen-level susceptibility scores remained unchanged at 0.25 (IQR = 0.25–1, *P* = .06) before and after EAC. Forty of

Table 1. Characteristics of FUVL80-999 and FUVL≥1000 populations

|	FUVL80-999	FUVL≥1000
N	10	47
Female, n (%)	5 (50)	34 (72)
Age in years, median [IQR]	43.4 [38.8–54.4]	41.1 [30.0–49.4]
Pill count in percent, median [IQR][a,b]	100 [100–100]	98 [96–100]
WHO stage, n (%)[c]		
1	4 (40)	14 (30)
2	4 (40)	18 (38)
3	2 (20)	12 (26)
4	0 (0)	2 (4)
VL before EAC in copies/mL, median [IQR]	3515 [1852–15424]	13971 [5973–25869]
VL after EAC in copies/mL, median [IQR]	476 [318–600]	9738 [4644–21033]
ART regimen, n (%)		
AZT/3TC/EFV	4 (40)	11 (23)
AZT/3TC/NVP	3 (30)	13 (28)
TDF/3TC/EFV	3 (30)	19 (40)
TDF/3TC/NVP	0 (0)	4 (9)
Time on ART in years, median [IQR][d]	4.7 [4.0–5.3]	4.5 [2.5–6.5]
Days between VL before and after EAC, median [IQR][e]	100.0 [92.0–103.5]	105.0 [98.0–115.8]
CD4 cell count before EAC (median [IQR])	408 [250–569]	350 [208–466]

[a]Number of pills presumably taken since the last visit (ie, number of pills provided at last visit minus number of pills remaining in pill bottle) divided by the number of pills that should have been taken since the last visit, multiplied by 100%.

[b]Missing: 2 participants in FUVL≥1000 population.

[c]Missing: 1 participants in FUVL≥1000 population.

[d]Missing: 1 participants in FUVL80-999 population.

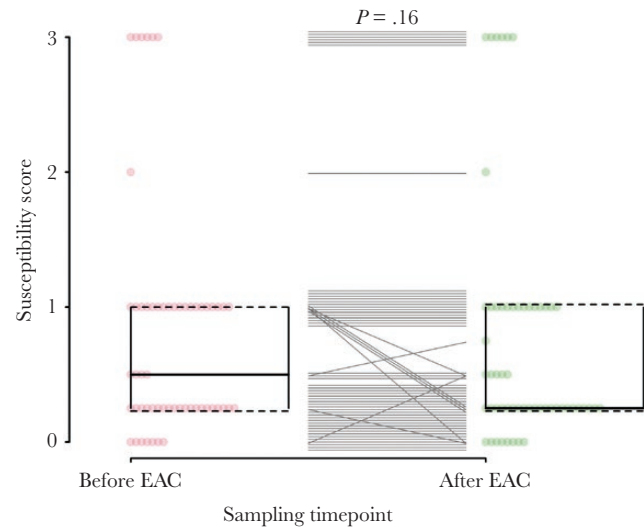[e]Missing: 3 participants in FUVL80-999 population and 5 participants in FUVL≥1000 population.
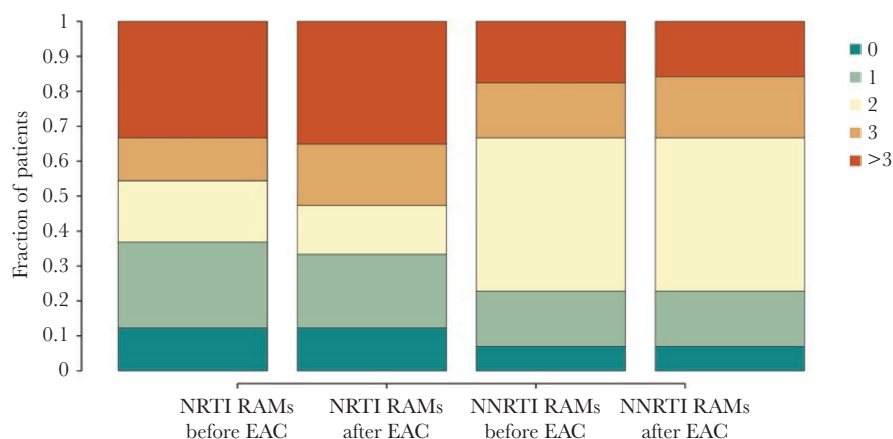
Figure 1. Fraction of participants with respective number of genomic positions with at least 1 major resistance-associated mutation (RAM) (with a variant frequency >5%) at respective time point[1]. [1] Observed major RAMs: M41L, A62V, K65R, D67N, K70E, K70R, V75I, F77L, Y115F, M184V, M184I, L210W, T215Y, T215F, K219Q, K219E, L100I, K101P, K101E, K103N, K103S, V106M, V108I, E138A, E138G, E138Q, Y181C, Y188C, Y188L, Y188H, V179L, G190A, G190S, H221Y, P225H, M230L, M230I, D30N. EAC, enhanced adherence counseling; NNRTI, nonnucleoside reverse-transcriptase inhibitor; NRTI, nucleoside reverse-transcriptase inhibitor.

the 47 individuals (85.1%) in this group experienced no change in susceptibility scores over the course of the EAC period, whereas 6 of 47 (12.8%) had a decreased score after EAC and 1 of 47 (2.1%) had an increased score after EAC (Supplementary Figure S2). The characteristics and RAMs of the 7 individuals experiencing a change in susceptibility are listed in Table 2. Only 6 of 47 (12.8%) individuals in the FUVL ≥1000 group were fully susceptible to their current regimen, and 28 of 47 (59.6%) harbored RAMs conferring resistance to all the drugs in their regimen already before EAC. In the FUVL80-999 group, the median susceptibility score did not change between the time point of the first (median = 1, IQR = 0.44–1) and the

follow-up (median = 1, IQR = 0.63–1, $P = .32$) VL test. In this group, the susceptibility score remained unchanged in 9 of 10 (90.0%) individuals and increased in 1 of 10 (10.0%) individual (Supplementary Figure S3). The characteristics and RAMs of the individual with changed susceptibility scores are listed in Table 2. None of the participants in FUVL80-999 group had HIV-1 that was fully susceptible to their regimen, and 3 of 10 (30.0%) harbored HIV-1 that was not fully susceptible to any of the drugs in their regimen.

Change of Variant Frequency During the Enhanced Adherence Counseling Period

We found major RAMs with a patient-level variant frequency of >5% at 26 genomic positions (Supplementary Figure S4). Of these, mutations in 12 (M41L, A62V, K65R, D67N, K70E/R, V75I, F77L, Y115F, M184I/V, L210W, T215F/Y, K219E/Q) positions are associated with resistance to NRTIs, 13 (L100I, K101E/P, K103N/S, V106A/M, V108I, E138A/G/Q, V179L, Y181C/I, Y188C/H/L, G190A/S, H221Y, P225H, M230L/I) are associated with NNRTI resistance, and 1 (D30N) is associated with protease inhibitor resistance. Although mean variant frequencies did not change significantly over the course of the EAC period, we observed a trend ($P < .1$) towards increased variant frequency after EAC for A62V, V106A/M, and K219E/Q and towards decreased frequency for G190A/S. The stratified analyses of the FUVL ≥1000 and the FUVL80-999 group are shown in Supplementary Figure S5 and Supplementary Figure S6, respectively.

DISCUSSION

This study examines the evolution of HIVDR during the WHO-recommended EAC period in a resource-limited setting, thereby assessing the risk of further resistance

Figure 2. Susceptibility scores before and after enhanced adherence counseling ([EAC] N = 57). Gray lines connect samples of the same individual. Box plots indicate the median (bold horizontal line) and the interquartile range (dashed horizontal line). The *P* value was derived using sign test.

Table 2. Characteristics and RAMs of Participants With a Change in Susceptibility Scores Before Versus After EAC[a]

Population	ID	VL Before EAC [c/mL]	VL After EAC [c/mL]	ART Regimen	Susceptibility Score Before EAC	Susceptibility Score After EAC	Number of Fully Active Drugs Before EAC	Number of Fully Active Drugs After EAC	Major RAMs Before EAC (Frequency)	Major RAMs After EAC (Frequency)
FUVL80-999	1	1933	944	TDF/3TC/EFV	0	0.5	0	0	K65R (0.65), D67N (0.32), K70R (0.39), L100I (0.17), K103N (0.99), V108I (0.57), M184V (1), K219E (0.38), K219Q (0.62)	D67N (1), K70R (1), K103N (1), V108I (1), M184V (1), K219E (0.5), K219Q (0.5). No longer observed: K65R (0), L100I (0).
FUVL ≥1000	2	20 369	2747	AZT/3TC/EFV	1	0.5	1	0	K70R (0.18), K103N (0.7), K103S (0.3), V106M (0.32), V108I (0.68), M184V (1), H221Y (0.75)	D67N (0.09), K70R (0.75), K103N (0.24), K103S (0.76), V106M (0.87), V108I (0.11), M184V (1), K219Q (0.1), H221Y (0.11)
	3	28 595	12 156	AZT/3TC/NVP	1	0.25	1	0	K101E (1), M184V (1), G190A (1), T215Y (0.32)	K101E (1), M184V (1), G190A (1), T215Y (0.83)
	4	26 586	15 446	TDF/3TC/EFV	1	0.25	1	0	A62V (0.08), K65R (0.19), D67N (0.07), K103N (1), V106M (1), M184V (0.81), M184I (0.19)	A62V (0.72), K65R (0.74), K70E (0.22), K103N (0.94), V106M (0.94), Y115F (0.22), M184V (0.92). No longer observed: D67N (0), M184I (0).
	5	2603	2463	AZT/3TC/EFV	0.25	0	0	0	M41L (0.75), K103N (1), E138A (1), M184V (1), Y188L (1), L210W (0.43), T215Y (1)	M41L (0.96), K103N (1), E138A (1), M184V (1), Y188L (1), L210W (0.68), T215Y (1)
	6	3643	10 517	TDF/3TC/EFV	0.5	0.75	0	0	D67N (0.96), K70R (0.91), K103N (0.11), V108I (0.77), Y181C (0.93), M184V (0.86), G190A (0.9), K219Q (0.89)	D67N (1), K70R (0.51), K103N (0.12), V106M (0.48), V108I (0.39), Y181C (1), M184V (1), G190A (0.5), K219Q (1)
	7	20 7772	13 592	TDF/3TC/EFV	1	0.25	1	0	A62V (0.31), K65R (0.33), D67N (0.17), K103N (0.62), K103S (0.38), V106M (1), M184V (0.97)	A62V (1), K65R (1), K103N (0.39), K103S (0.61), V106M (1), M184V (1). No longer observed: D67N (0).
	8	7360	1271	AZT/3TC/NVP	1	0	1	0	D67N (0.98), K70R (0.36), K101E (0.98), V106M (1), E138A (1), M184V (1), K219Q (0.36)	D67N (0.88), K70R (0.83), K101E (0.98), V106M (1), E138A (1), M184V (1), K219Q (0.83)

Abbreviations: ART, antiretroviral therapy; AZT, zidovudine; EAC, enhanced adherence counseling; EFV, efavirenz; NVP, nevirapine; RAM, resistance-associated mutation; TDF, tenofovir disoproxil fumarate; VL, viral load; 3TC, lamivudine.

[a]Red and green indicate an increase and decrease, respectively, in measured variant frequency.

accumulation with this clinical standard of care. Previously, before EAC, we observed extremely high levels of resistance, and the majority (54.4%) of participants already harbored HIV-1 with at least partial resistance to all the drugs in their ART regimen. The median regimen-level susceptibility scores dropped nonsignificantly from 0.5 to 0.25 during the EAC period, indicating an increase in resistance. Of note, because RAMs that drop below the limit of detection may remain archived in the latent reservoir [23, 24], the increase in susceptibility scores observed in a small subset of participants might not correspond to an actual disappearance of drug resistance. The results reported here align with an observation by Kantor et al [25] of additional emergence of drug resistance in patients on second-line (lopinavir/ritonavir-based) ART after a median of 55 days.

This study is not without limitations. The number of included participants was limited by the availability of well documented patient samples (from the CART-1 study), and the sample size in the FUVL80-999 group was low. Furthermore, the CART-1 participants may not be representative of patients living in resource-limited settings today for several reasons. First, this study relies on samples from 2014 and does not include any patients on protease inhibitors or integrase strand-transfer inhibitors. Nevertheless, this study includes drugs that have been taken by the vast majority of people on ART in resource-limited settings, and that may remain relevant for those for whom integrase inhibitors are contraindicated. Second, participants received their first-ever VL test in the context of this study despite having been on ART for a median of 4.6 years, meaning that they most likely had already spent extensive time on a failing regimen. Indeed, the rate of resuppression observed in CART-1 is higher than in another study from Uganda with participants receiving their first-ever VL test after extensive time on ART [26], but it is low compared with data from a recent systematic review, which showed that approximately half of the PWH with an initial unsuppressed VL resuppressed after adherence counseling [8]. Today, routine (generally yearly) VL monitoring might lead to accelerated clinical action, although resistance levels are lower than those observed in this study. On the one hand, identifying viremia earlier could lead to higher rates of resuppression than observed in CART-1. On the other hand, it is possible that the further emergence of resistance and loss of clinically relevant drug options would be even greater in patients with new treatment failure due to HIVDR.

Taking into consideration that only 17 of 93 (18.3%) participants in the CART-1 study resuppressed to <80 c/mL, and considering the high levels of drug resistance among those who did not resuppress, our results indicate that the vast majority of participants with initial viremia in CART-1 would have benefitted from a rapid switch to second-line ART, provided sufficient counseling was given around the time of the switch to promote high onward adherence. Of note, all participants with partial resuppression to

80–999 c/mL had extensive drug resistance, making it unlikely that they will achieve long-term suppression on first-line ART and calling into question the WHO cutoff of 1000 c/mL for viral suppression. It is also worth noting that in CART-1, among 110 patients with an initial ≥1000 c/mL, 17 never received a second VL: however, 5 were switched after their first VL, 3 died (AIDS-related), and 9 were lost to follow-up. These 2 latter groups may have fared better upon an immediate intervention. In settings with high loss to follow-up and where patients may struggle to pay transport fees to the healthcare facility, additional clinical visits and delays in switching to second-line ART may lead to increased attrition from care. In Lesotho, in routine clinical care, only 40% of patients with an unsuppressed VL receive a follow-up VL within 6 months, and only 25% of those receiving a follow-up VL either resuppress or are switched to second-line ART within 3 months [9]. Apart from leading to higher morbidity and mortality [10–14], such delays increase the time window in which HIV-1 drug resistance can emerge and onward transmission of resistant HIV-1 may take place.

CONCLUSIONS

In summary, we observed extremely high levels of HIVDR already before EAC, as well as a slight further increase in resistance mutations and a nonsignificant decrease in regimen-level susceptibility at follow-up. These results indicate that most participants would have benefitted from earlier detection of treatment failure followed by a rapid switch to second-line ART, and they suggest that the current treatment algorithm bears a certain risk of further accumulation of drug resistance, potentially jeopardizing future treatment options. However, these findings should be balanced against the benefit EAC has in achieving resuppression without regimen switch and thus avoiding unnecessary treatment changes.

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