Towards achieving the third “90”of the 90-90-90 global HIV targets in sub-Saharan Africa

Adherence, drug resistance and molecular diagnostics

Inzaule, S.C.

Global efforts for HIV/AIDS have now been galvanized around achieving the UNAIDS 90-90-90 targets (90% of people living with HIV knowing their status, 90% of those infected receiving ART, and 90% of those on ART having sustained viral suppression) which if achieved by 2020, would lead to HIV epidemic control by 2030. There are multiple factors challenging the attainment of these targets. This thesis focus on the challenge for achieving the third '90' goal for viral suppression with specific focus on adherence, drug resistance and need for better molecular diagnostics in sub-Saharan Africa.

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Towards achieving the third "90" of the 90-90-90 global HIV targets in sub-Saharan Africa

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Dit proefschrift is tot stand gekomen in het kader van het 'International Doctorate in Transdisciplinary Global Health Solutions' - Erasmus Mundus Joint Doctorate Trans Global Health Programme, met als doel het behalen van een gezamenlijk doctoraat. Het proefschrift is voorbereid in de Faculteit der Geneeskunde van de Universiteit van Amsterdam en in het Institut de Salut Global de Barcelona (ISGlobal) van de Universitat de Barcelona

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Abbreviations

3TC	lamivudine
AZT	azidothymidine (also referred to as ZDV)
ADR	acquired drug resistance
AIDS	acquired immunodeficiency syndrome
AIGHD	Amsterdam Institute for Global Health and Development
ART	combination antiretroviral therapy
ARV	antiretroviral (drug)
ATV-r	ritonavir boosted atazanavir
CHAI	Clinton Health Access Initiative
CRF	circulating recombinant form
d4T	stavudine
DNA	deoxyribonucleic acid
DBS	dried blood spots
DRM	drug resistance mutation
DRV-r	ritonavir-boosted darunavir
DTG	dolutegravir
EFV	efavirenz
FTC	emtricitabine
GFATM	Global Fund to Fight AIDS, Tuberculosis and Malaria
GRT	genotypic resistance test
GSS	genotypic sensitivity scores
HIV	human immunodeficiency virus
HIVDR	HIV drug resistance
HIV TrePS	HIV treatment response prediction system
HR	hazard ratio
IAS-USA	International Antiviral Society-USA
INSTI	integrase strand transfer inhibitors
JCRC	Joint Clinical Research Centre
LMIC	low- and middle-income country
LPV-r	ritonavir-boosted lopinavir
LTFU	loss to follow-up
MARCH	Monitoring Antiretroviral Resistance in Children
MTCT	mother-to-child transmission of HIV-1
NFV	nelfinavir
NGS	next-generation sequencing

NNRTI	non-nucleoside reverse transcriptase inhibitor
NRTI	nucleoside reverse transcriptase inhibitor
NVP	nevirapine
OR	odds ratio
PASER	PanAfrican Studies to Evaluate Resistance
PAU	prior ARV use
PEPFAR	US President's Emergency Plan for AIDS Relief
PCR	polymerase chain reaction
PDR	pretreatment drug resistance
PI	protease inhibitor
PMA	point mutation assay
PMTCT	prevention of mother-to-child transmission of HIV-1
POC	point of care
RAL	raltegravir
RNA	ribonucleic acid
RT	reverse transcriptase
sd-NVP	single-dose nevirapine as PMTCT
TAM	thymidine analogue mutation
VAS	visual analogue scale
VL	HIV viral load
WHO	World Health Organization
ZDV	zidovudine (also referred to as AZT)

Chapter 1

General introduction

The AIDS pandemic

In 1981, a new disease outbreak characterized by opportunistic infections and Kaposi sarcoma was reported in a small number of homosexual men in US cities[1,2], later denoted as acquired immunodeficiency syndrome (AIDS). Two years later, a retrovirus, now known as human immunodeficiency virus (HIV) was discovered as the causative agent, which can be transmitted sexually, through blood-to-blood contact and perinatally from mother to child.[3] During the next three decades, more than 78 million people would become infected with HIV, with 39 million dying from AIDS related causes.[4] Today, the disease is mainly concentrated in sub-Saharan Africa with nearly 67% of people living with HIV/AIDS residing in this region.[4]

History of antiretroviral treatment scale-up in low- and middle-income countries

Antiretroviral treatment in the early years of the HIV/AIDS pandemic was limited to only a few drugs usually given as mono or dual therapy. This proved to be sub-optimal, offering only a limited survival benefit, due to the rapid emergence of drug-resistant HIV variants i.e. viruses which carry mutations that allow them to replicate in the presence of the drug that once inhibited their growth.[5–7]

The breakthrough came in 1996, when studies demonstrated that a cocktail of triple antiretroviral drugs (ARVs) targeting at least two different stages in the HIV replication cycle (figure 1) could achieve durable viral suppression and delay the development of resistance.[8–11] This strategy involving the use of two nucleoside transcriptase inhibitors (NRTIs) as a backbone and a core agent from another class of ARVs would become the cornerstone for HIV treatment, known as combination antiretroviral therapy (ART). By the year 2000, a dramatic decline in HIV/AIDS related morbidity and mortality had been observed in western countries, attributed to the expanded use of ART.[12]

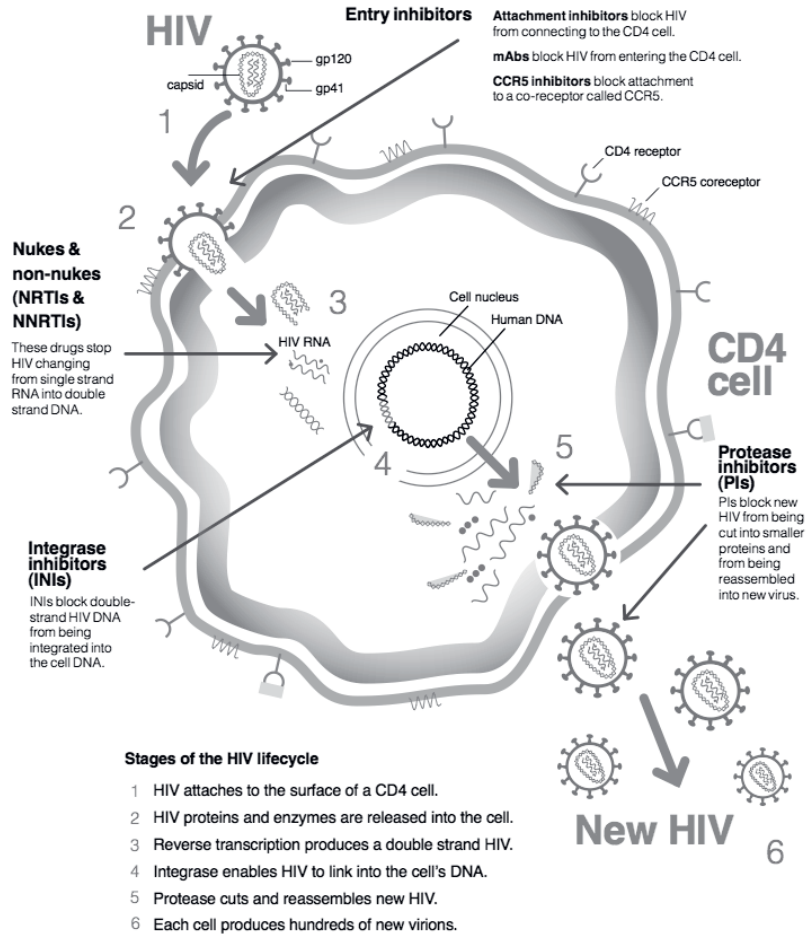

Figure 1: HIV life-cycle and drug targets (source http://i-base.info/guides/starting/hiv-life-cycle)

Despite these major advances, access to ART was highly limited in low and middle-income countries (LMIC). The initial expansion had been hindered by concerns of high costs, weak health care systems to support the delivery of complex ART and speculations of HIV drug resistance (HIVDR) anarchy due to inadequate health systems.[13,14] With millions of new HIV infections annually, the general view among the donor community was to prioritize prevention, which was deemed more feasible and cost-effective than treatment.[15]The turnaround came in the early 2000s, as a result of increased political will, activism from the global community and the availability of low-cost generic drugs.

In 2001, the UN general assembly held a special session of HIV/AIDS, which led to the creation of Global Fund to Fight AIDS, TB and Malaria (GFATM). This together with other initiatives such as the US President's Emergency Plan for AIDS Relief (PEPFAR) helped to create steady financial mechanisms to support ART delivery in LMIC. In 2003, WHO launched the 3 by 5 initiative aimed at reaching 3 million patients in LMIC on ART by the end of 2005, a target that would be achieved in 2007.[16]

To circumvent the lack of supportive infrastructure and funding constraints, WHO adopted a public health approach for ART delivery as opposed to the resource-intensive, individualized-based care, used in resource-rich settings.[17] This involved standardized treatment protocols that promoted the use of simplified regimens, simplified clinical monitoring, limited laboratory support, a decentralized system and provision of care by lower cadre staff. Ideally, patients would be treated using three sequential ART standardized regimens constituting a non-NRTI (NNRTI) core drug with 2 NRTIs as the first-line regimen and, upon treatment failure, switched to a ritonavir boosted protease inhibitor (PI) + 2 new or recycled NRTIs as the second-line regimen followed by integrase strand transfer inhibitor (INSTI) + 1 or 2 new or recycled NRTIs as third-line ART.

By the end of 2017, an estimated 60% of the 25.7 million people living with HIV/AIDS in Sub-Saharan Africa were receiving ART under the WHO public health approach.[18] This is in contrast to <10% a decade earlier.[19]

As treatment becomes widely available, the global attention has been shifting from the initial goal to prevent early mortality to that of improving lifelong survival and improved quality of life. Progress has also been made to provide more efficacious and safer drugs with simplified dosage. Since 2016, efforts have also been made to accelerate access to newer more efficacious, well tolerated, easy dosing drugs for example dolutegravir- a second generation INSTI.

In addition, treatment has also gained an additional role in preventing new HIV infections following studies showing a reduced risk of onward transmission in virologically suppressed patients. [20,21] Based on this premise, the UNAIDS in 2014 issued ambitious targets for the global HIV response, the 90-90-90 goals (90% of all HIV infected people knowing their status, 90% of all people diagnosed with HIV infection placed on ART, and 90% of all people receiving ART achieving viral suppression), which if implemented by 2020 and scaled up to 95-95-95 thereafter, would lead to HIV epidemic control by 2030. Modeling showed that achieving

these targets would result in a 90% decline in new infections and 80% decline in AIDS related mortality compared to 2010 levels. However, the achievement of the third goal is threatened primarily by rising levels of HIVDR, challenges in medication adherence, poor retention in care, and limited access to optimal treatment monitoring diagnostics[22–25].

HIV drug resistance

HIVDR emerges as a consequence of the virus' high error-prone replication mechanism[26,27] coupled with rapid replicative turnover.[28,29] This rapid evolution generates a swarm of closely related but genetically diverse viral population (*quasispecies*) within an individual, which includes a large number of potentially drug-resistant mutant variants.[30]

The drug-resistant mutants have a reduced replicative fitness and are usually outgrown by the fitter wild-type (drug susceptible) viruses. The presence of antiretroviral drugs, particularly at sub-optimal concentration, exerts a selective pressure that favors the growth of pre-existing drug-resistant mutants resulting in acquired drug resistance (ADR). Patients with ADR can transmit the resistant virus to other individuals, referred to as transmitted drug resistance (TDR). Pre-treatment drug resistance (PDR) is used to define drug resistance mutations detected in HIV-infected persons before starting ART, resulting from either TDR or prior exposure to antiretroviral drugs (e.g. short-course prophylaxis for prevention of mother-to-child transmission (PMTCT), pre or post-exposure prophylaxis, or first-line ART restarters after earlier treatment interruption).[24] Several factors contribute to the development of HIVDR and they mainly affect the person's ability to take their medication as required (adherence). These can be broadly classified into drug-related factors, viral factors, patient-related factors and programmatic factors (Figure 2)

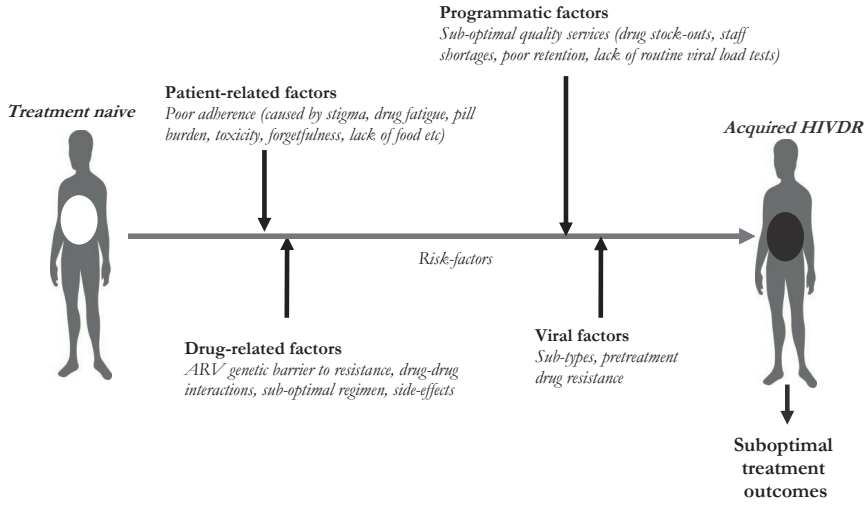

Figure 2: Factors associated with HIV drug resistance

Drug-related factors

The ease at which the virus becomes resistant to a drug depends on the drugs' genetic barrier to resistance, defined as the number of mutations needed for the virus to overcome the drug-selective pressure.[31,32] A drug with a high genetic barrier will require the virus to develop multiple mutations to overcome the drug's inhibitory ability, for instance ritonavir-boosted PIs and the second-generation INSTI dolutegravir. [31,32] On the contrary, drugs with low genetic barrier may lose their effectiveness even in the presence of only 1 mutation, for instance most NNRTIs. [31,32]

Drug side effects could lead to poor adherence and increase the risk of developing resistance.[33] Drug-drug interactions can also reduce the effective concentrations of antiretroviral drugs to levels that select for drug resistant viruses. For example, rifampin used for TB prevention/treatment has been shown to significantly reduce the levels of PIs, INSTIs and some NNRTIs.[34]

Viral factors

The degree of transmission of drug resistant mutants depends on the fitness cost that the mutation imposes on the virus. Mutations conferring a high fitness cost tend to reduce the viral replicative capacity of the mutant variant and are less likely to be transmitted compared to those with low fitness cost. [35,36]

Overall, the presence of PDR increases the risk that the patient develops virological failure and that the virus further accumulates drug resistance mutations.[37] The risk of having PDR is particularly high in infants who become infected through mother-to-child transmission due to exposure to the sub-optimal doses of maternal antiretroviral regimens ingested during breastfeeding and/or the sub-therapeutic regimens given to them as prophylaxis. [22,38] The propensity for development of resistance may also vary across subtypes which could be driven in part by differences in viral polymorphisms.[39]

Patient-related factors (adherence associated factors)

Life-long optimal adherence to ART is required so as to achieve sustained viral suppression and improve immunological and clinical outcomes. Patients need to take their medication every day and at a consistent time, failure of which increases the risk of developing virological failure, acquisition and transmission of drug resistant variants, disease progression and death[40–42]. As with other chronic diseases, sustaining high-level adherence to self-administered medication is a challenge and is affected by several factors including treatment related factors; treatment fatigue, high pill burden, more frequent dosing, drug side effects, patient related factors; stigma, irregular dosing schedule, forgetfulness, non-disclosure, lack of treatment supporter and being an adolescent or young adult, structural factors like food insecurity and lack of transport for timely access to medication[43–46]

Programmatic factors

Weaknesses in the health care system can also facilitate the emergence of HIVDR at the population level.[47] Fragile supply chain systems can lead to drug stock-outs and subsequent treatment interruptions or dispensing of suboptimal ART regimens or unfavorable substitutions all of which impacts on the patient's adherence.

Shortages in human resource may affect the quality of services impacting adherence and retention.

Laboratory monitoring of ART effectiveness

Due to cost constraints, initial WHO guidelines recommended using immunological and clinical markers for ART monitoring.[17] Clinical monitoring entails the assessment of new or recurrent clinical symptoms while immunological monitoring involves the assessment of CD4 T cell counts; the key immune markers for HIV disease progression.

These however proved poor surrogates and have been associated with late detection of treatment failure, accumulation of drug resistance mutations, subsequently depleting future treatment options and increasing the risk of transmission of drug resistant variants at the population level.[48] Moreover, use of clinical-immunological monitoring has poor specificity for identifying persons with virological failure and thus lead to unnecessary treatment switches to a limited, more expensive, next-line of treatment.[48]

Virological monitoring

Virological monitoring involves the quantification of viruses circulating in the blood and is considered to be the gold standard. This is because a rise in detectable virus precedes the decline in CD4 counts or occurrence of clinical symptoms during natural prognosis of HIV as well as during treatment failure.[49,50]

To improve treatment monitoring, WHO guidelines from 2013 started recommending routine viral-load tests in LMICs.[51] In addition, evidence from studies showing reduced risk of HIV transmission in virally suppressed patients has further necessitated the need to monitor population-based viral load. This shift in paradigm has created an increase in demand for viral load testing, but the infrastructure requirements for expansion remains daunting. To fill this gap, new technologies including point-of-care tests for increased decentralized access[52] and use of dried blood spots (DBS) to ease specimen collection and transportation, are being explored, but the implementation is still a challenge.[52,53]

Drug resistance testing

Similarly, WHO under the public health approach, recommended resistance testing only for population-based surveillance to monitor the effectiveness of treatment and provide information for population-level decision making. This is contrary to the practice in resource-rich settings where individualized resistance tests are routinely used to optimize treatment in both naive and patients failing ART. Recently, however the need for HIVDR for individualized resistance testing in LMICs has increasingly been recognized and current WHO guidelines recommend their use in patients failing on PI based second-line treatment. [54] In addition to treatment optimization, individualized resistance tests help prevent unnecessary switches to third-line or salvage regimens, which are expensive and usually not available for programs in these settings. However wide access to resistance tests is limited by high costs and technical requirements and their cost-effectiveness is still debatable.[55–58] There are

also concerns that promoting their use might divert resources from high priority areas like expanding access to treatment and viral load tests.

RESEARCH OBJECTIVES

Aim

The aim of this thesis is to assess optimal strategies that promote sustained viral suppression in HIV treatment in sub-Saharan Africa

Objectives

- To identify determinants of long-term sustained viral suppression
- To define pre-treatment drug resistance among HIV-infected infants
- To assess novel, affordable diagnostics for improving ART monitoring

Research setting

The Pan-African Studies to Evaluate Resistance (PASER) was established in 2006 with the aim of developing regional capacities for population-based assessment of acquired and transmitted HIVDR.[59] The PASER network comprised a partnership of 13 clinical sites, laboratories and research groups in Kenya (2), Nigeria (1), South-Africa (3), Uganda (3), Zambia (3) and Zimbabwe (1), with initial coordination from PharmAccess Foundation and later the Amsterdam Institute for Global Health and Development. PASER has been a key partner of the WHO strategy on the prevention and assessment of HIVDR.[60]

The PASER cohort enrolled a total of ~ 3000 participants, 240 from each of the participating sites.[59] This included ~2700 participants on first-line ART and 250 on second-line ART. Participants were followed up for a period of between 24-72 months and received care according to national guidelines, which were in accordance to WHO HIV treatment guidelines. The median age of those who initiated first-line ART was 36.8 years (IQR 31.3–42.6) and 58% were female. Majority of the participants had advanced disease; 60% with WHO stage III or IV disease and 37% had pre-treatment CD4 counts of less than 100 cells/µl.
The median age of the participants who started on second-line ART was 38.6 years (IQR 32.9–44.2) and 50% were female. The median CD4 count was 125 cells/µl (IQR 46–196) while 48% had WHO stage III or IV disease.

The studies in this thesis extend on previous findings from the PASER-M cohort and two spinoff studies: Monitoring Antiretroviral Resistance in Children (MARCH), a pediatric cohort in Uganda and Nigeria, and Affordable Resistance Testing in Africa (ARTA), which was a public-private consortium that aimed to develop a more affordable HIVDR test. Previously published findings include:

- Overall PDR prevalence in adults (2007-2009) was found to be 5.6%, ranging from a low of 1.1 in Pretoria, South Africa to 12.3% in Kampala, Uganda .[61]
- PDR was shown to have important clinical impact including slower recovery of CD4 cells, increased risk of treatment failure, and increased risk of treatment switches.[37]
- PDR in children <12 years (2010-2012) in Uganda (3 clinic sites) and Nigeria (1clinic site) was found to be between 10 and 15%, with a higher prevalence in children reporting previous exposure to PMTCT (35.7%) and in those whose PMTCT exposure was unknown (15.6%).[62,63]
- PDR in children was equally associated with increased risk of virological failure and ADR.[62,64]
- Participants switching to second-line ART had high levels of virological suppression despite predicted partial activity of the co-administered NRTIs in second-line regimens.[65]
- One in five participants failing second-line ART were had resistance to PI drugs highlighting the need for increasing access to third-line regimen in these settings.[66]
- Use of viral load tests for ART monitoring was shown to potentially prevent unnecessary treatment switches and accumulation of drug resistance mutations.[48]
- The low cost ARTA viral load(Viral Failure assay) and the Ultralight HIVDR in-house assay (reverse transcriptase-based, one-step HIV drug resistance genotyping test) were shown to have good accuracy when compared to commercial assays and could thus be used to improve access to better treatment monitoring in LMICs.[67,68]

This thesis builds on these studies with a special emphasis on optimized strategies to achieve the third 90' UNAIDS target for sustained viral suppression.

OUTLINE OF THE THESIS

Part I: Determinants of long-term sustained viral suppression

Part I of this thesis describes studies that assess the determinants of long-term successful ART outcomes in HIV-infected patients in sub-Saharan Africa. First, we assess the effect of prior ARV exposure on PDR and on virological response, based on a prospective analysis of PASER-M cohort participants initiating first-line ART (**Chapter 2**). Next, we assess the impact of pretreatment drug resistance on virological response on different first-line regimens (**Chapter 2**). In **Chapter 3,** we assess the durability of first-line ART and reasons for treatment modifications in a cohort of HIV patients in western Kenya. In **Chapter 5**, we present the findings of a qualitative study assessing the barriers and facilitators of long-term ART adherence in adolescents and adults in Uganda. In **Chapter 6**, we describe the findings of a nationwide study describing the emergence of untreatable, drug-resistant HIV among patients failing on second-line ART, due to exhaustion of treatment options in Kenya. In **Chapter 7**, we assess the prevalence and patterns of primary resistance to INSTIs across diverse HIV-1 subtypes circulating in sub-Saharan Africa, in preparation for wide-scale rollout of dolutegravir-based ART in the region. **Chapter 8** is a Viewpoint that discusses the need for caution in presenting dolutegravir as an overall solution to the rise of HIVDR in LMICs and in addition proposes a multipronged approach.

Part II: Burden of PDR among HIV-infected infants

In **Part II**, we describe the prevalence of PDR in infants and assess potential mitigation strategies. We first describe a high prevalence of K65R mutation in treatment naive but PMTCT-exposed HIV-infected infants (**Chapter 9**). Next, we present the findings of a nationwide survey of prevalence of PDR in HIV-infected infants in Nigeria (**Chapter 10**). In **Chapter 11** we discuss potential ways to prevent PDR in infants citing the limited treatment options available for these children despite the need for lifelong ART.

Part III: Affordable diagnostics for improved ART monitoring

In **Part III** we assess potential affordable diagnostics to improve ART monitoring in sub-Saharan Africa. In **Chapter 12,** we assess the impact of the WHO-

recommended stringent viral load thresholds on DBS-based viral load assays and ART programs. In **Chapter 13**, we evaluate a low-cost DBS-based in-house HIVDR genotypic assay for use in LMIC. In Chapters 14 and 15, we further review existing low-cost HIVDR diagnostic technologies (**Chapter 14**) and the operational aspects (**Chapter 15**) needed to improve access in LMIC. In **Chapter 16**, we evaluate the clinically relevant thresholds necessary for the operationalization of low-cost next-generation sequencing assays in LMIC.

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Part I

Determinants of long-term sustained viral suppression

Chapter 2

Previous antiretroviral drug use compromises standard first-line HIV therapy and is mediated through drug-resistance

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ABSTRACT

In ART programs in sub-Saharan Africa, a growing proportion of HIV-infected persons initiating first-line antiretroviral therapy (ART) have a history of prior antiretroviral drug use (PAU). We assessed the effect of PAU on the risk of pre-treatment drug resistance (PDR) and virological failure (VF) in a multicountry cohort of HIV-infected adults initiated on a standard non-nucleoside reverse transcriptase inhibitor (NNRTI)-based first-line ART. Multivariate logistic regression was used to assess the associations between PAU, PDR and VF (defined as viral load ≥400 cps/mL). Causal mediation analysis was used to assess the proportion of the effect of PAU on VF that could be eliminated by intervening on PDR.

Of 2737 participants, 122 (4.5%) had a history of PAU. Participants with PAU had a 7.2-fold (95%CI 4.4-11.7) risk of carrying PDR and a 3.1-fold (95%CI 1.6-6.1) increased risk of VF, compared to antiretroviral-naïve participants. Controlling for PDR would eliminate nearly half the effect of PAU on the risk of VF.

Patients with a history of PAU are at increased risk of ART failure, which is to a large extent attributable to PDR. These findings support the recent WHO recommendations for use of differentiated, non-NNRTI-based empiric first-line therapy in patients with PAU.

INTRODUCTION

In low and middle-income countries (LMICs), antiretroviral treatment (ART) regimens to treat HIV-1 infections are standardized under the WHO-defined public health approach[1]. Although reliable data are limited, ART programs in sub-Saharan Africa have reported that between 10 and 25% of first-line ART initiators have previously used antiretroviral drugs, either because they re-started ART after disengaging from care, or they used short-course antiretrovirals through prevention of mother-to-child transmission (PMTCT) programs, or pre- or post-exposure prophylaxis[2].

People with previous antiretroviral drug use (denoted PAU) are at an increased risk of having drug-resistant HIV before starting ART (denoted pre-treatment drug resistance, PDR)[2,3], which impairs response to standard non-nucleoside reverse transcriptase inhibitor (NNRTI)-based first-line ART[4–6]. However, for patients with PAU, few studies to date have evaluated the response to standard first-line ART or optimal management[7]. The vast majority of LMICs provide standard first-line therapy regardless of antiretroviral history or PDR testing[1,8].

This study aimed to investigate the effects of PAU on PDR and virological failure (VF) in a multi-country cohort in sub-Saharan Africa, and the extent of which this effect could be eliminated by intervening on PDR.

METHODS

Study design and population

The Pan-African Studies to Evaluate Resistance Monitoring (PASER-M) study was a prospective multi-country cohort including 13 sites in 6 countries (Kenya, Nigeria, South Africa, Uganda, Zambia, Zimbabwe), as profiled elsewhere[5], conducted between 2007 and 2014. All participants were followed up according to local standard-of-care guidelines. The present study included all participants who initiated first-line ART containing an NNRTI plus two NRTIs. Retrospective viral load (VL) testing was performed before ART initiation and annually after ART initiation. Participants provided written informed consent at study enrolment. The study was approved by the appropriate research ethics committees at all collaborating sites and the

Amsterdam UMC of the University of Amsterdam, Institutional Review Board. The study was performed in accordance with relevant guidelines and regulations.

Virological analysis

VL and PDR were retrospectively measured at either of two reference laboratories in Uganda and South Africa[5]. Sanger sequencing of the *pol* gene was performed if VL ≥1000 cps/ml using in-house assays. PDR was defined as the presence of ≥1 major drug resistance mutation (DRM) included in the International Antiviral Society–USA mutation list of December 2017 that are associated with any NRTI or the NNRTIs nevirapine or efavirenz [9], plus the revertant mutations at codon 215 (A/C/D/E/N/S/V)[10].

Statistical analyses

Logistic regression analysis

Multivariate logistic regression with robust standard errors to account for clustering of observations within sites was used to assess the association between PAU and PDR and VF at month 12, defined as VL ≥400 cps/mL or a switch to second-line ART due to treatment failure up to 12 months. PAU was defined both as a dichotomous and a categorical variable according to type as follows: none, ART (standard triple ARV combinations), single-dose nevirapine (sdNVP) for PMTCT, or other ARV combinations (including mono/dual therapy). Models were adjusted for potential confounders, which were selected stepwise from the following list of independent variables: age, sex, country, calendar year of treatment initiation, type of NNRTI and NRTI, PDR, pretreatment VL and CD4 cell count, and the 12 months average of 30-day self-reported adherence. Subsequently, we investigated PDR as a potential effect modifier of the association between PAU and VF by including an interaction term in the model and stratifying the model according to the presence/absence of PDR.

Causal mediation analysis

We also investigated PDR as a potential intermediate on the causal pathway of the association between PAU and VF using causal mediation analysis (Figure 1)[11]. We calculated: 1) the *proportion mediated*, a measure that determines how much of the effect of the exposure (PAU) on the outcome (VF) is due to the effect of the exposure (PAU) on the intermediate (PDR). Proportion mediated is calculated as the ratio of natural indirect effect (NIE, effect of PDR on VF assuming all participants had PAU) to the total effect (TE), where $TE = NIE + NDE$ (natural

direct effects, effect of PAU on VF assuming PDR prevalence is similar in persons with/without PAU); 2) the *proportion eliminated,* a measure that determines the effect of the exposure (PAU) on the outcome (VF), that could be eliminated by intervening on the intermediate (PDR). Conceptually, this is the scenario where each patient receives a fully active ART regimen, either empirically or guided by PDR testing; therefore, by intervening on PDR we could eliminate a part of the effect of PAU on VF. Proportion eliminated is calculated as , *TE – CDE(m=0)/TE, where CDE (controlled direct effects) is the* effect of PAU on VF while fixing the intermediate PDR (m) to level 0. The causal mediation analysis was done using the paramed syntax in Stata with log-linear regression, assuming interaction[12].

Sensitivity analyses

We performed the following sensitivity analyses to confirm the robustness of the associations: (1) We used a higher VL threshold of ≥1000 cps/mL to define VF (WHO definition[1]); (2) We assessed the effect of PAU on acquired drug resistance (ADR) at 12 months for patients with VF ≥1000 cps/mL; (3) We further elucidated the effects of PDR on VF by restricting its definition to the presence of NNRTI-resistance (NNRTI-PDR); (4) We assessed the longer-term effect of PAU on VF (up to 24 months follow-up);

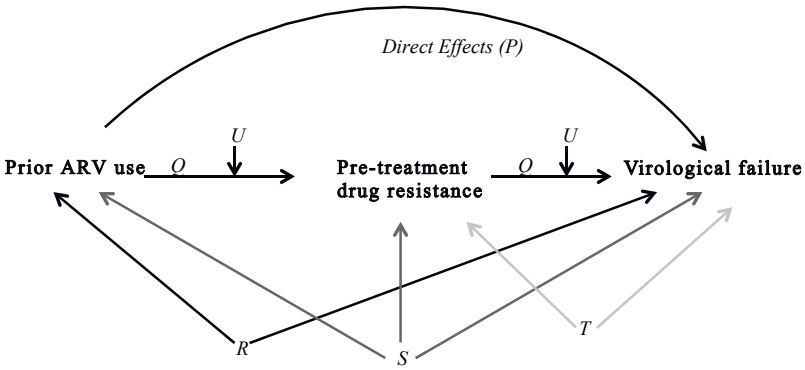

Figure 1: Direct aclyclic graph (DAG) showing the relationship between prior ARV use, pre-treatment drug resistance and virological failure.

P represents direct effects of prior ARV use on virological failure. Q represents indirect effects of prior ARV use on virological failure mediated through pre-treatment drug resistance. R represents confounders of the association between prior ARV use and virological failure: age and sex. S represents confounders of the association between pre-treatment drug resistance and virological failure that are affected by prior ARV use: pre-treatment viral load, CD4 cell counts and adherence. T represents confounders of the association between pre-treatment drug resistance and virological failure not included in S: type of initial antiretroviral treatment (NNRTI and NRTI) initiated and calendar year of treatment initiation. U represents unmeasured confounders. We note that the DAG is only a simple illustration and this may exclude complex relationships interacting between prior ARV use/pre-treatment drug resistance/virological failure and their confounders.

RESULTS

Patient characteristics

Of the 2737 participants initiating ART, 122 (4.5%) had a documented history of PAU and 2615 (95.5%) were antiretroviral-naive; 23 (0.8%) participants were excluded because information on PAU was missing (Table 1). PAU comprised: ART (50%, 61/122), sdNVP (32%, 39/122), and other ARV combinations (18%, 22/122) (Table S1). Compared to antiretroviral-naïve participants, those with PAU were more likely to be female (5.9% vs 2.6%, p<0.001), younger (median age 34.7 years [IQR 29.1-40.5] vs 37.0 [IQR 32.0-43.3], p<0.001) with higher pre-treatment CD4 cell counts (median 177 [IQR 147-202] vs 133 [IQR 62-203] cells/µl, p=0.0017) (Table 1). Females were more frequently exposed to sdNVP (2.6% vs 0%, p<0.001) and other ARV combinations (1.3% vs 0.2%, p=0.001), but not to ART (2.2% vs 2.4%, p=0.813). The proportion of participants who had an average adherence level ≥95% did not differ between patients with (86.5%) or without (86.4%) PAU (p=0.977).

Table 1: Baseline characteristics of patients with and without prior ARV use

Characteristic	Prior ARV use N=122	ARV naive N=2592	P-value
Age (years) Median IQR	34.7 (29.1-40.5)	37.0 (32.0-43.3)	<0.001
Sex, n (%)			
Female	29 (23.8)	1108 (42.8)	<0.001
Male	93 (76.2)	1484 (57.3)	
VL (log10) Median (IQR)	4.9 (4.1-5.6)	5.2 (4.4-5.6)	0.240
CD4 (Log10) Median (IQR)	177 (147-202)	133 (62-203)	0.0017
WHO clinical stage			0.275
I/II	54 (44.3)	1019 (39.3)	
III/IV	68 (55.7)	1573 (60.7)	
Type of initial NNRTI			
EFV	73 (59.8)	1545 (59.6)	0.964
NVP	49 (40.2)	1046 (40.4)	
Type of initial NRTI backbone			
TDF+XTC	43 (35.3)	866 (33.4)	0.790
d4T+3TC	29 (23.8)	695 (26.8)	
ABC+3TC	2 (1.6)	66 (2.6)	
ZDV+3TC	48 (39.3)	964 (37.2)	
†ART adherence			0.977
≥95%	96 (86.5)	2044 (86.4)	
<95%	15 (13.5)	322 (13.6)	

Data are presented as n (%), unless stated otherwise
3TC, lamivudine; ABC, abacavir; ART, antiretroviral therapy; d4T, stavudine; EFV, efavirenz; NVP, nevirapine; TDF, tenofovir; VL: viral load; XTC, lamivudine or emtricitabine; ZDV, zidovudine;
†Mean adherence measured as 30-day self-reported adherence over 12 months;

Effect of PAU on PDR

2557/2714 (94.2%) participants had a PDR test performed, of whom 144 (5.6%) had PDR, with 115/2442 (4.7%) in antiretroviral-naïve participants and 29/115 (25.2%) among those with PAU (p<0.001) (Figure 2). The proportion of participants who carried any DRM, NNRTI-resistance, NRTI-resistance and dual-class resistance was: 29.1%, 27.3%, 12.7%, 10.9%, respectively, after ART; 28.6%, 14.3%, 19.1%, 4.8%, respectively, after other ARV combinations; 18.0%, 12.8%, 5.1%, 0.0%, respectively, after sdNVP; and 4.7%, 3.6%, 2.1%, 1.1%, respectively, for those who were antiretroviral-naive. In the adjusted analysis, the odds of PDR was 7.2-fold (95%CI 4.4-11.7; p<0.001) higher in participants with PAU, compared to those who were antiretroviral-naive; and varied with the type of PAU: aOR 15.1 (95%CI, 5.3-42.5; p<0.001) after other ARV combinations, 9.1 (95%CI 4.8-17.2; p<0.001) after ART, and 3.3 (95%CI 1.4-8.1; p=0.008) after sdNVP (Table 2).

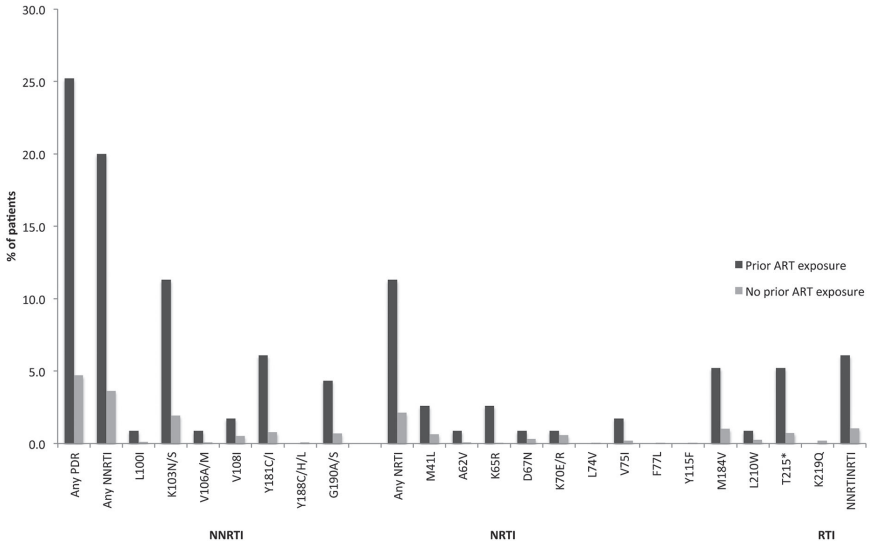

Figure 2: Patterns of drug resistance mutations in participants with and without prior ARV use

Of all DRMs detected, 25.2% occurred in the participants with PAU. The proportion of participants who carried NNRTI, NRTI and dual NNRTI+NRTI resistance was 20.0%, 11.3% and 6.1% respectively for those with PAU and 3.6%, 2.1%, 1.1% for antiretroviral-naïve participants respectively.

Table 2: Baseline characteristics of patients with and without prior ARV use

Characteristic	Prior ARV use N=122	ARV naive N=2592	P-value
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WHO clinical stage			0.275
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ZDV+3TC	48 (39.3)	964 (37.2)	
†ART adherence			0.977
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<95%	15 (13.5)	322 (13.6)	

Data are presented as n (%), unless stated otherwise
3TC, lamivudine; ABC, abacavir; ART, antiretroviral therapy; d4T, stavudine; EFV, efavirenz; NVP, nevirapine; TDF, tenofovir; VL: viral load; XTC, lamivudine or emtricitabine; ZDV, zidovudine;
†Mean adherence measured as 30-day self-reported adherence over 12 months;

Effect of PAU on VF

Multivariable regression analysis

200/2031 (9.8%) participants who had a VL test result at 12 months experienced VF while 9 other patients had been switched to second-line treatment before 12 months. Of these 20/86 (23.3%) patients had PAU and 190/1953 (9.7%) were antiretroviral-naive (p≤0.001). In the adjusted analysis, the odds of VF was 3.1-fold (95%CI 1.6-6.1; p=0.001) higher in participants with PAU, compared to those who were antiretroviral-naive; and varied with the type of PAU: aOR 3.1 (95%CI 0.9-11.0; p=0.088) after sdNVP, 3.9 (95%CI 1.6-9.1; p=0.002) after ART, and 1.4 (95%CI 0.5-5.0; p=0.562) after other ARV combinations (Table 2).

The association between PAU and VF was similar when using a higher VL threshold

(aOR 3.5, 95%CI 1.-7.4; p=0.001) (sensitivity analysis 1; Table S2). Genotypic results were available for 130/182 participants with a viral load >1000cps/mL at 12 month, 95 (73%) of which had one or more major drug-resistance mutations. Compared with antiretroviral-naive participants, people with PAU had an increased risk of ADR (aOR 3.5, 95% CI 1.3–9.2; p=0·010) (sensitivity analysis 2; Table S3).

Effect modification by PDR

In the regression model with interaction term, there was no evidence that PDR was an effect modifier of the association between PAU and VF (p for interaction=0.485) (Table 2). Similarly, there was no evidence for interaction when we used a higher VL threshold (p for interaction=0.432) (sensitivity analysis 1; Table S2) or when PDR was restricted to NNRTI-PDR (p for interaction=0.451) (sensitivity analysis 3; Table S4).

Causal mediation by PDR

Table 1 summarizes the causal mediation analysis. The total effects of PAU on the risk of VF was aOR 4.8 (95%CI 2.3-10.0; p<0.001). The proportion of the effect of PAU on VF mediated through PDR was 38%. The controlled direct effects of PAU, when fixing PDR=0, remained statistically significantly associated with VF (aOR 2.7, 95%CI 1.5-5.0; p=0.002). The proportion of the effect of PAU on VF that could be eliminated by intervening on PDR was 48%.

Compared to the main analysis, a VL threshold of >1000 cps/ml to define VF resulted in a slight reduction in the *proportion mediated* (33%) and an increase in the *proportion eliminated* (51%%) (sensitivity analysis 1; Table S2), and a restricted definition of NNRTI-PDR resulted in a similar *proportion mediated* (36%) and an increase in the *proportion eliminated* (63%) (sensitivity analysis 3; Table S4).

Long-term effect of PAU on VF *(sensitivity analysis 4)*

192/1838 (10%) participants who had a VL test result at 24 months experienced VF while 53 other patients had been switched to second-line treatment before 24 months. Of these 25/73 (34.2%) patients had PAU and 243/1818 (13.4%) were antiretroviral-naive (p≤0.001). In the adjusted analysis, the odds of VF was 4.3-fold (95%CI 2.3-8.2; p<0.001) higher in participants with PAU, compared to those antiretroviral-naive; and varied with the type of PAU: aOR 2.9 (95%CI 0.8-10.4; p=0.110) after sdNVP, 6.7 (95%CI 3.0-14.6; p<0.001) after ART, and 3.0 (95%CI 1.0-9.0; p=0.055) after other ARV combinations (Table S5).

Causal mediation analysis showed that the proportion of the effect of PAU on VF mediated through PDR was 24% and the proportion of the effect of PAU on VF that could be eliminated by intervening on PDR was 29%.

DISCUSSION

This prospective study among HIV-infected adults in sub-Saharan Africa starting first-line NNRTI-based ART found that persons who had a history of PAU, i.e. ART or sdNVP for PMTCT, were seven times more likely to have PDR, and three times more likely to experience VF within the first year of NNRTI-based ART, compared to those who were antiretroviral-naïve at ART initiation.

A causal mediation analysis provided two important additional insights. First, the pathway through the intermediate PDR was estimated to explain about 38% of the operation of the effect of PAU on VF. Conceptually, this means that the higher VF rates found in patients with PAU could partially be attributed to the presence of PDR, predominantly associated with the drug class of NNRTIs. We speculate that the residual effect of PAU could partially be attributed to unmeasured NNRTI-resistant minority variants, since the limited sensitivity of Sanger-based sequencing to detect minority virus populations may have resulted in an underestimation of the total effect of PDR. NNRTI-resistant minority variants have previously been shown to be associated with VF[13], with higher impact among patients with PAU[14,15].

Second, if we could eliminate the effect of the intermediate PDR on VF, the effect of PAU on VF is estimated to be reduced by half or more. Conceptually, this means that the use of an alternative fully-active first-line regimen in patients with PAU could half the number of failures that are attributable to PAU. This could be achieved by adopting either of two strategies in patients with PAU: the use of individualized PDR testing to guide the choice of first-line treatment, or a change of standard first-line regimen that is non-NNRTI-based (e.g. dolutegravir). The latter option has the advantage of addressing the potential residual impact of PAU on VF due to unmeasured minority resistant variants in absence of more sensitive resistance tests.

Previous studies on this topic are limited. Across seven WHO-led national surveys in LMICs, PDR was found to be considerably higher among persons with PAU

(22%) than among antiretroviral-naive people (8%)[2]. A cross-sectional study in Nigeria found that patients with PAU were four times more likely to experience VF when initiated on NNRTI-based therapy[7].

Our findings emphasize the importance of thorough assessment of previous antiretroviral history before ART initiation, and the use of non-NNRTI-based empiric first-line therapy (e.g. based on the integrase-inhibitor dolutegravir) in line with the latest WHO guidelines (July 2017)[8]. Our findings also provide further support to lifelong ART in childbearing women (PMTCT option B+) to avoid the risks associated with cycles of ART stopping and re-starting.

Our findings suggest that potential interventions to eliminate the effect of PDR (i.e. by providing an alternative fully-active first-line ART) could significantly reduce the risk of VF attributable to PAU. This impact is particularly substantive in reducing early VF during the first year of ART (48% risk reduction up to 12 months). However, in the longer term the impact may be more modest (29% risk reduction up to 24 months). These findings suggest that in the longer term the influence of PDR may be waning and that other factors explain the continuous impact of PAU on the risk of VF. We hypothesize that unaddressed factors associated with the initial default from care (hence the presence of PAU) may be undermining succesful adherence to long-term treatment, underscoring the need for enhanced adherence interventions for patients with PAU.

Strengths of the study were its prospective design, large sample, and the availibility of combined data on PAU, PDR and virological outcomes. The setting of routine ART programs enhanced the generalizibility of the results to other LMICs.

Study limitations were the lack of detailed PAU histories, precluding an in-depth analysis of attributes such as adherence, dosage, timing and duration, and the use of patient self-report and medical records to document PAU histories, with potential for recall and desirability bias[16]. This could have resulted in overall underestimation in the effect of PAU on VF. These limitations highlight the importance of enhancing electronic patient information systems that can link patient data across ART delivery sites.

In conclusion, patients with a history of PAU in African ART programs are at increased risk of ART failure, which is to a large extent attributable to the presence of PDR. To help meet the third of the UNAIDS global targets (i.e. ensuring viral

suppression in 90% of people on ART), the choice of first-line ART regimens should be guided by a thorough assessment of antiretroviral history. Patients with PAU should receive differentiated, non-NNRTI-based empiric first-line therapy as recommended by the latest WHO guidelines.

Competing interests

All authors declare that they have no conflict of interest.

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Authors' contributions

TFRW is the PASER principal investigator. CMK, MS, ASA, MW, MJ, PI, KM, TSB, KCES, TFRW and RLH established the cohort and supervised data collection. CMK and WS supervised laboratory testing. SCI, TFRW and RLH conceived the study. SCI performed the statistical analyses, with assistance from RLH. SCI and RLH drafted the manuscript. All authors provided valuable input to interpretation of the data and critically reviewed the paper for important intellectual content. All authors reviewed and approved the final version of the manuscript.

Data availability

All necessary data is included in the manuscript but any additional information is available upon request.

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SUPPLEMENTARY MATERIAL

Table S1: Details on types of antiretroviral regimens in patients with prior ARV use (N=122)

Type of previous ARV use	N (%)
Single-dose NVP for PMTCT	39 (32)
ART	61 (50)
d4T+3TC+NVP	17 (28)
d4T+3TC+EFV	10 (16)
ZDV+3TC+NVP	8 (13)
ZDV+3TC+EFV	5 (8)
TDF+FTC+EFV	3 (5)
Unspecified	18 (30)
Other ARV combinations*	22 (18%)
Dual-therapy PMTCT	18 (82%)
Dual therapy non-PMTCT	3(14%)
Mono-therapy§	1(5%)

3TC, lamivudine; ART, antiretroviral therapy; ARV, antiretroviral; d4T, stavudine; EFV, efavirenz; NVP, nevirapine; TDF, tenofovir; ZDV, zidovudine;
*Type of regimen was missing or not recorded;
§Included non-sdNVP for PMTCT

Table S2: Effect of prior ARV use on virological failure, defined by higher VL threshold (≥1000 cps/mL) (Sensitivity analysis #1)

	N	Events	Unadjusted OR (95%CI)	P-value	Adjusted OR (95%CI)	P-value
Effect of prior ARV use on VF [a]						
Any prior ARV use						
No	1953	163	1.0		1.0	
Yes	86	19	2.9 (1.7-5.2)	<0.001	3.5 (1.7 -7.2)	0.001
Logistic regression model including interaction term (prior ARV use * PDR) [b]						0.432
Effect of prior ARV use on VF, PDR as intermediate on causal pathway [c]						
Causal mediation analysis						
Natural direct effects (NDE)			2.3 (1.2-4.2)	0.008	3.1 (1.6-5.8)	0.001
Natural indirect effects (NIE)			1.5 (0.9-2.4)	0.109	1.9 (1.0-3.4)	0.043
Controlled direct effect (CDE)			2.2 (1.1-4.5)	0.008	2.8 (1.3-5.8)	0.007
Total effects (TE)[d]			3.4 (1.8-6.5)	<0.001	5.7 (2.6-12.4)	<0.001
Proportion mediated PM = NIE / TE = 33%						
Proportion eliminated PE = (TE – CDE) / TE =51%						

Abbreviations: ART, antiretroviral combination therapy; ARV, antiretroviral; PDR, pretreatment drug resistance; VF, virological failure; NIE, natural indirect effects; NDE, natural direct effects; CDE, controlled direct effects; TE, total effects

[a] Adjusted for age, sex, pre-treatment CD4 counts, pre-treatment viral load, PDR, type of ART, calendar year of ART initiation and adherence

[b] Adjusted for age, sex, pre-treatment CD4 counts, pre-treatment viral load, type of ART, calendar year of ART initiation and adherence

[c] Adjusted for age, sex, pre-treatment CD4 counts, pre-treatment viral load, type of ART, calendar year of ART initiation and adherence

[d] odds ratio for TE i.e. $OR^{TE}=OR^{NDE}*OR^{NIE}$

Table S3: Effect of prior ARV use on acquired drug resistance (Sensitivity analysis #2)

	N	Events	Unadjusted OR (95%CI)	P-value	Adjusted OR (95%CI)	P-value
Effect of prior ARV use on ADR [a]						
Any prior ARV use						
No	1883	85	1.0		1.0	
Yes	77	10	3.2 (1.6-6.4)	0.001	3.5 (1.3 -9.0)	0.011

Abbreviations: ADR, acquired drug resistance; ARV, antiretroviral [a] Adjusted for age, sex, pre-treatment CD4 counts, pre-treatment viral load, type of ART, calendar year of ART initiation and adherence. Analysis included 12 months viral load + acquired drug resistance as a composite outcome variable

Table S4: Associations between prior antiretroviral drug use, pre-treatment NNRTI drug resistance and virological failure (Sensitivity analysis #3)

	N	Events	Unadjusted OR (95%CI)	P-value	Adjusted OR (95%CI)	P-value
Logistic regression model including interaction term (prior ARV use * NNRTI PDR [b]						0.451
Effect of prior ARV use on VF, NNRTI-PDR as intermediate on causal pathway [c]						
Natural direct effects (NDE)			2.1 (1.2-3.7)	0.015	2.8 (1.5-5.2)	0.001
Natural indirect effects (NIE)			1.7(1.0-2.9)	0.066	2.3 (1.1-4.4)	0.019
Controlled direct effect (CDE)			2.0 (1.0-3.8)	0.048	2.4 (1.2-4.9)	0.014
Total effects (TE)[d]			3.5 (1.7-7.3)	<0.001	6.4 (2.5-16.1)	<0.001
Proportion mediated PM = NIE / TE =36%						
Proportion eliminated PE = TE – CDE / TE =63%						

Abbreviations: ART, antiretroviral combination therapy; ARV, antiretroviral; NNRTI, non-nucleoside reverse transcriptase inhibitor; PDR, pretreatment drug resistance; VF, virological failure, NIE, natural indirect effects; NDE, natural direct effects; CDE, controlled direct effects; TE, total effects;

[a] Adjusted for age, sex, pre-treatment CD4 count, pre-treatment viral load, country, WHO clinical stage, calendar year of ART initiation
[b] Adjusted for age, sex, pre-treatment CD4 count, pre-treatment drug resistance, type of ART, calendar year of ART initiation and adherence
[c] Adjusted for age, sex, pre-treatment CD4 count, pre-treatment viral load, type of ART, calendar year of ART initiation and adherence
[d] Odds ratio for TE i.e. $OR^{TE}=OR^{NDE}*OR^{NIE}$

Table S5: Effect of prior ARV use on virological failure, up to 24 months follow-up (Sensitivity analysis #4)

	N	Events	Unadjusted OR (95%CI)	P-value	Adjusted OR (95%CI)	P-value
Effect of prior ARV use on VF [a]						
Any prior ARV use						
No	1818	243	1.0		1.0	
Yes	73	25	3.2 (2.0-5.3)	<0.001	4.3 (2.3 -8.2)	<0.001
Type of prior ARV use						
None	1818	243	1.0		1.0	
ART	36	16	4.9 (2.7-8.8)	<0.001	6.7(3.0-14.6)	<0.001
sdNVP	26	6	1.9 (0.6-6.1)	0.259	2.9 (0.8-10.4)	0.110
Other	11	3	2.4 (0.8-7.1)	0.107	3.0 (1.0-9.0)	0.055
Logistic regression model including interaction term (prior ARV use * PDR) [b]						0.767
Effect of prior ARV use on VF, PDR as intermediate on causal pathway [c]						
Causal mediation analysis						
Natural direct effects (NDE)			3.0 (1.7-5.1)	<0.001	3.8 (2.1-6.9)	<0.001
Natural indirect effects (NIE)			1.3 (0.9-2.0)	0.223	1.4(0.9-2.4)	0.164
Controlled direct effect (CDE)			3.1 (1.7-5.6)	<0.001	3.9(2.0-7.4)	<0.001
Total effects (TE)[d]			3.8 (2.1-7.0)	<0.001	5.5 (2.8-11.0)	<0.001
Proportion mediated PM = NIE / TE = 24%						
Proportion eliminated PE = (TE – CDE) / TE = 29%						

Abbreviations: ART, antiretroviral combination therapy; ARV, antiretroviral; PDR, pretreatment drug resistance; VF, virological failure; NIE, natural indirect effects; NDE, natural direct effects; CDE, controlled direct effects; TE, total effects

[a] Adjusted for age, sex, pre-treatment CD4 counts, pre-treatment viral load, PDR, type of ART, calendar year of ART initiation and adherence

[b] Adjusted for age, sex, pre-treatment CD4 counts, pre-treatment viral load, type of ART, calendar year of ART initiation and adherence

[c] Adjusted for age, sex, pre-treatment CD4 counts, pre-treatment viral load, type of ART, calendar year of ART initiation and adherence

[d] Odds ratio for TE i.e. $OR^{TE}=OR^{NDE}*OR^{NIE}$

Chapter 3

Does tenofovir-containing first-line ART mitigate the impact of pretreatment NNRTI drug resistance?

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In an HIV treatment-as-prevention trial in South Africa, Durache et al[1] report the remarkable finding that the presence of NNRTI-associated pre-treatment drug-resistance (PDR) did not impair virological response to fixed-dose tenofovir/emtricitabine/efavirenz (Atripla). This is an important contrast with most past studies that found that NNRTI-associated PDR was associated with a 2-3-fold increased risk of virological failure (VF)[2–9], although most patients in those studies received a thymidine analogue backbone (zidovudine/stavudine), with efavirenz or nevirapine.[2,3] The authors attributed their finding to the better potency of efavirenz compared to nevirapine, and an Editorial Comment added the advantage of the similar half-lives of the Atripla components, making it less likely for resistance to emerge during missed doses.[10]

In our previous analysis in the Pan-African Studies to Evaluate Resistance (PASER-M) cohort, we reported that patients with NNRTI-PDR had a >2-fold increased risk of VF, compared to patients with susceptible virus[4,7]. Based on the hypothesis by Durache et al, we extended this to a stratified analysis by type of first-line regimen. We defined PDR as (1) NNRTI, NRTI or dual-class NNRTI+NRTI resistance, based on 2017-IAS-USA mutation list; (2) Stanford genotype susceptibility scores (GSS; v8.7) <3 of the prescribed first-line regimen. We defined VF as a single viral-load (VL) ≥50, 400 or 1000 cps/ml measured at month 12. We assessed the association between PDR and VF using logistic regression, while adjusting for key confounders.

Of 2,737 participants initiating first-line ART, 1,941 had data on PDR and 12 month VL. Median age was 37.0 years (IQR31.7-43.1), 59.8% were women, and 56.4% had an overall mean VAS adherence of ³95%. Initial regimens contained tenofovir+lamivudine/emtricitabine (xtc) (33%), with efavirenz (27.3%) or nevirapine (5.7%), or a non-tenofovir, thymidine analogue backbone+xtc (67%), with efavirenz (29.8%) or nevirapine (37.1%). 1838 (94.7%) patients had no PDR, 79 (4.1%) had NNRTI-PDR only, 44 (2.3%) had NRTI-PDR and 24 (1.2%) had dual-class NNRTI+NRTI-PDR. 84 (4.4%) patients initiated a first-line regimen with GSS<3. VF was present in 335 (17.3%), 199 (10.3%) and 172 (8.9%) participants at VL≥50, 400 and 1000 cps/ml thresholds, respectively.

Participants who had PDR defined as GSS<3, NNRTI only, or dual-class NNRTI+NRTI who received non-tenofovir/xtc with efavirenz or nevirapine, had an increased risk of VF, compared to those without PDR. However, this risk was not increased for participants who received tenofovir/xtc/efavirenz, whereas there was a borderline association for participants who received tenofovir/xtc/nevirapine (Table 1).

Table 1: Effect of pre-treatment drug resistance on risk of virological failure after 12 months of ART

Characteristic	N	No. of events	aOR (95%CI)	P-value	No. of events	aOR (95%CI)	P-value	No. of events	aOR (95%CI)	P-value
			VF≥50 cps/ml			VF≥400 cps/ml			VF≥1000 cps/ml	
TDF-containing ART	641	109			58			50		
GSS <3	30	7	1.56 (0.35-6.90)	0.560	5	2.31(0.36-14.73)	0.377	5	2.94 (0.56-15.35)	0.200
IAS NNRTI	31	8	1.82 (0.43-7.66)	0.412	6	2.91(0.56-15.15)	0.205	6	**3.76 (0.88-16.02)**	**0.073**
IAS NRTI	21	4	0.87 (0.29-2.62)	0.799	2	0.89 (0.22-3.36)	0.839	2	1.18 (0.34-4.14)	0.796
IAS NNRTI+NRTI	12	3	1.09 (0.23-5.12)	0.915	2	1.48 (0.35-6.32)	0.595	2	1.97 (0.54-7.22)	0.305
TDF/XTC/EFV	530	81			40			34		
GSS <3	22	4	1.40 (0.22-9.03)	0.725	3	2.81 (0.25-31.50)	0.437	3	3.72 (0.38-36.37)	0.259
IAS NNRTI	23	5	1.75 (0.32-9.43)	0.517	4	3.63 (0.46-28.31)	0.218	4	4.96 (0.70-35.08)	0.109
IAS NRTI	17	3	0.99 (0.20-4.90)	0.989	1	0.74 (0.05-9.90)	0.817	1	1.01 (0.08-12.75)	0.995
IAS NNRTI+NRTI	8	2	1.31 (0.09-18.58)	0.840	1	1.53 (0.72-32.62)	0.786	1	2.05 (0.11-38.95)	0.633
TDF/XTC/NVP	111	28			18			16		
GSS <3	8	3	1.69 (0.81-3.53)	0.160	2	1.68 (0.98-2.88)	0.059	2	**2.62 (1.01-6.75)**	**0.047**
IAS NNRTI	8	3	1.69 (0.81-3.53)	0.160	2	1.68 (0.98-2.88)	0.059	2	**2.62 (1.01-6.75)**	**0.047**
IAS NRTI	4	1	0.91 (0.31-2.67)	0.858	1	1.81 (0.65-5.07)	0.257	1	2.95 (0.70-12.46)	0.142
IAS NNRTI+NRTI	4	1	0.91 (0.31-2.67)	0.858	1	1.81 (0.65-5.07)	0.257	1	2.95 (0.70-12.46)	0.142
Non-TDF-containing	1299	226			141			122		
ART	55	26	**4.93 (2.51-9.68)**	**<0.001**	19	**5.25 (2.00-14.07)**	0.001	18	**5.74 (2.13-15.51)**	**0.001**
GSS <3	49	26	**6.53 (3.11-13.72)**	**<0.001**	20	**7.24 (3.00-17.43)**	<0.001	18	**6.97 (2.76-17.61)**	**<0.001**
IAS NNRTI	24	6	1.42 (0.69-2.91)	0.340	4	1.52 (0.43-5.39)	0.515	4	1.78 (0.49-6.42)	0.380
IAS NRTI	13	5	3.04 (0.91-10.13)	0.070	4	**3.83 (1.24-11.87)**	0.020	4	**4.32 (1.44-12.94)**	**0.009**
IAS NNRTI+NRTI										
Non-TDF/XTC/EFV	578	88			59			50		
GSS <3	17	7	**5.82 (1.96-17.27)**	**0.002**	6	**6.88 (1.75-27.08)**	**0.006**	5	**5.25 (1.53-17.99)**	**0.008**
IAS NNRTI	17	8	**6.79 (2.62-17.57)**	**<0.001**	6	**8.18 (2.69-24.84)**	**0.006**	5	**4.48 (1.20-16.72)**	**0.026**
IAS NRTI	7	1	0.84 (0.09-8.15)	0.890	1	1.09 (0.15-8.15)	0.930	1	1.17 (0.16-8.26)	0.878
IAS NNRTI+NRTI	3	1	1.78 (0.14-23.35)	0.661	1	1.93 (0.20-18.86)	0.571	1	1.86 (0.20-17.64)	0.590
Non-TDF/XTC/NVP	721	138			82			72		
GSS <3	38	19	**5.36 (1.90-15.11)**	**0.001**	13	**5.60 (1.15-27.29)**	**0.033**	13	**6.61 (1.40-31.34)**	**0.017**
IAS NNRTI	32	18	**7.37 (2.46-22.09)**	**<0.001**	13	**8.07 (1.79-36.29)**	**0.005**	13	**9.56 (2.26-40.51)**	**0.002**
IAS NRTI	17	5	1.78 (0.71-4.46)	0.219	3	1.85(0.43-8.03)	0.411	3	2.12 (0.47-9.65)	0.329
IAS NNRTI+NRTI	10	4	3.48 (0.73-16.54)	0.117	3	4.81 (1.18-19.66)	0.029	3	5.47 (1.46-20.53)	0.012

Abbreviations: aOR, adjusted odds ratio; EFV, efavirenz; XTC, lamivudine or emtricitabine; IAS, International Antiviral Society mutation list; GSS, genotypic sensitivity scores; NRTI, nucleoside reverse transcriptase inhibitor; NNRTI, non-NNRTI; NVP, nevirapine;
Odds ratios were adjusted for sex, type of initial NNRTI and NRTI, WHO clinical stage, BMI, calendar year of ART initiation, mean VAS adherence, and prior ARV exposure,pre-ART viral load and CD4 cell count.

Participants with NNRTI-PDR only who received a tenofovir-containing regimen had an increased risk of VF at the VL≥1000 cps/ml threshold (with borderline statistical significance p=0.073), and the risk was not increased at the ≥50 and ≥400 cps/ml thresholds.

In conclusion, our analysis corroborates the finding that NNRTI-PDR may impact less on tenofovir/xtc/efavirenz than on thymidine analogue-based regimens especially with nevirapine. Nonetheless, it remains difficult to disentangle the possible beneficial effects of tenofovir, efavirenz, and fixed-dose combinations with similar drug half-lives. Given that 2 other studies have produced conflicting data[2,3], it is premature to argue that Atripla is equally efficacious for patients with or without NNRTI-PDR.

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Authors' contributions

TFRW is the PASER principal investigator. RLH, TFRW and RP conceived the study. SCI perfomed the statistical analysis. SCI and RLH drafted the manuscript. All authors provided valuable input to interpretation of the data and critically reviewed the paper for important intellectual content. All authors reviewed and approved the final version of the manuscript.

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Chapter 4

Incidence and predictors of first line antiretroviral regimen modification in Western Kenya

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ABSTRACT

Background

Limited antiretroviral treatment regimens in resource-limited settings require long-term sustainability of patients on the few available options. We evaluated the incidence and predictors of combined antiretroviral treatment (cART) modifications, in an outpatient cohort of 955 patients who initiated cART between January 2009 and January 2011 in western Kenya.

Methods

cART modification was defined as either first time single drug substitution or switch. Incidence rates were determined by Poisson regression and risk factor analysis assessed using multivariate Cox regression modeling.

Results

Over a median follow-up period of 10.7 months, 178 (18.7%) patients modified regimens (incidence rate (IR); 18.6 per 100 person years [95% CI: 16.2-21.8]). Toxicity was the most common cited reason (66.3%). In adjusted multivariate Cox piecewise regression model, WHO disease stage III/IV (aHR; 1.82, 95%CI: 1.25-2.66), stavudine (d4T) use (aHR; 2.21 95%CI: 1.49-3.30) and increase in age (aHR; 1.02, 95%CI: 1.0-1.04) were associated with increased risk of treatment modification within the first year post-cART. Zidovudine (AZT) and tenofovir (TDF) use had a reduced risk for modification (aHR; 0.60 95%CI: 0.38-0.96 and aHR; 0.51 95%CI: 0.29-0.91 respectively). Beyond one year of treatment, d4T use (aHR; 2.75, 95% CI: 1.25-6.05), baseline CD4 counts ≤350 cells/mm3 (aHR; 2.45, 95%CI: 1.14-5.26), increase in age (aHR; 1.05 95%CI: 1.02-1.07) and high baseline weight >60kg aHR; 2.69 95% CI: 1.58-4.59) were associated with risk of cART modification.

Conclusions

Early treatment initiation at higher CD4 counts and avoiding d4T use may reduce treatment modification and subsequently improve sustainability of patients on the available limited options.

INTRODUCTION

Access to antiretroviral therapy in resource-constrained setting has increased tremendously since the WHO, 3 by 5 strategy initiative in 2005[1]. Currently about 6.2 million people in sub-Saharan Africa are on treatment reflecting an antiretroviral treatment (ART) coverage of about 56% [2]. While the challenge for complete coverage still holds, critical benefits have been achieved with reductions in morbidity, mortality and a general improvement in the quality of life [3, 4]. The sustainability of these gains is crucial especially in times of stagnating high HIV burden and the need to match the huge investments against HIV in resource constrained settings [5]. Current concerns towards sustainability include durability of potent and well-tolerated first-line regimen, resistance issues and the availability of more potent but less expensive second and third-line regimens [6].

Drug intolerability has been cited as the main reason as to why patients either modify or discontinue regimen [7-14]. While this may be a global concern, the situation in affluent countries is bearable owing to the treatment options available [15]. This is in contrast to the situation in resource-constrained settings where treatment regimens are limited and thus there are few options for patients experiencing drug intolerance [6]. Of the 24 FDA approved antiretroviral drugs in the six available classes, only 6 from three classes are commonly in use in resource-limited settings due to cost constraints [16]. This also limits the availability of second and third-line drugs for patients experiencing treatment failure.

The frequency of treatment modification reported in resource limited setting is fairly high and ranges from 8.3 to 78.4% for switch and 13.7-21% for discontinuation [8, 11, 14, 17]. The reported high levels of treatment modification may pose a challenge to treatment programs impacting on the overall cost of ART and limiting good patient prognosis. Due to these constraints maximizing the duration of patients on initial first-line regimen and optimizing the use of well-tolerated drugs are important.

In this study, we describe the rates, reasons and factors predictive of first-line antiretroviral treatment modification from an adult cohort, at a large HIV outpatient clinic in western Kenya.

METHODS

Study design, site and patients

We conducted a retrospective cohort study at Jaramogi Oginga Odinga teaching and referral hospital (JOOTRH): the largest referral hospital in western Kenya. The hospital is located in the southwest part of the country bordering Lake Victoria and serves an area with some of the worst health indicators in the country, including high prevalence of HIV infection (15.4%, which is greater than twice that of the national 7.1% prevalence)[18, 19]. Since 2003, the hospital provides comprehensive HIV care at no cost, as part of the national ART program through a joint effort with Columbia University (MTCT-plus program), Government of Kenya and the U.S. Centers for Disease Control (CDC).

Included in this analysis were non-pregnant adults of >15 years, who initiated first-line regimen between 1st January 2009 and 31st January 2011, and had at least one follow-up visit record. During the study period, the WHO 2006 guidelines for adolescents and adults adopted by MOH-NASCOP were in use [20]. The first-line regimen consisted of the NRTI backbone zidovudine (AZT) or stavudine (d4T) or tenofovir (TDF), with lamivudine (3TC) and either Nevirapine (NVP) or Efavirenz (EFV). Patients were initiated on treatment when they either had CD4 counts of ≤200 cells/mm3 or when they had WHO stage IV disease. They would then be followed up for 2 weeks after initiating treatment, monthly if stable and six months thereafter. During the visits, clinicians would collect the patient's demographics, clinical and pharmacological information in standardized optical character reader forms, which were then transcribed into the KEMRI/CDC HIV implementation science service program (HISS) electronic database, designed mainly for data management and program evaluation. Quality control for the stored data was done at regular intervals. At the time of registration, patients were given unique identifiers different from those in the patient support centers for concealment purposes.

Study outcomes and variables definitions

The primary outcome in this analysis was time to first combined antiretroviral treatment (cART) modification, defined as the time from treatment initiation to change of one or more antiretroviral drugs used as part of the initial first-line cART. Reasons for treatment modification were based on those documented by the clinician, usually as, toxicity, treatment failure (defined as immunological

failure, according to WHO 2006 guidelines as CD4 counts decrease of 50% from the on treatment peak value, or a persistent CD4 count lower than 100 cells or fall of CD4 counts to pre-therapy baseline or clinical failure defined as new or recurrent WHO stage IV condition), non-adherence, or others. In case the documented reason was recorded as "others", further chart review at the patient support center clinic, was done to identify the exact documented reason.

Independent variables assessed were mainly demographic and clinical in nature and included age at treatment initiation, gender, baseline CD4 counts, baseline WHO clinical stage, type of NNRTI treatment in the regimen (NVP vs EFV) and the type of NRTI backbone (AZT or TDF or d4T). Baseline parameters were assessed at cART initiation, which was also the entry point for the participants in this study.

Statistical analysis

Baseline patient characteristics were described using percentages for categorical data and median and inter-quartile ranges for continuous data. Incidence rates were calculated as the number of events over the person years of follow-up and the confidence intervals obtained from Poisson distribution. Drug specific incidence rates were determined as rate per persons initiating the specific drug. Kaplan-Meier analyses were used to estimate the time to first cART modification. Patients were censored at the time of event or at their last clinical follow-up visit.

Cox proportional hazards models were used to determine factors associated with cART modification. Due to violation of proportionality of hazards (PH), piecewise Cox regression models were fitted in at ≤12 months and >12 months which were time periods corresponding to the time at which the hazards were proportional. Predictor variables assessed included gender, age at treatment initiation, baseline weight, CD4 counts (obtained at closest date to treatment initiation, usually taken 6 months prior or after cART initiation), WHO stage, and the patient's cART regimen i.e. (NVP vs. EFV), (AZT vs. d4T/TDF), (TDF vs. AZT/d4T), (d4T vs. AZT/TDF). Information on baseline CD4 was missing for 178 patients (10.6% for those with cART modification and 20.5% for those who sustained treatment). The missing CD4 data was imputed by multiple imputation using chain equations (MICE) [21]. Prediction equation included WHO staging, baseline weight, age at treatment initiation, gender, time to treatment modification, treatment modification status and first-line regimens. Before imputation, continuous variables were normalized using square root transformation for age and log-transformation for baseline

weight. A total of 10 imputed data sets were generated.

Variables significant at univariate analysis (P< 0.10) were included in the multivariate models. Estimates of hazard coefficients were derived through averaging of the 10 iterations and appropriate standard errors calculated using the Rubin's rules [21, 22].

We also assessed factors associated with specific reasons of treatment modification grouped as toxicity and contraindication (TB treatment and other drug contraindications) for which there was sufficient data to conduct the sub-analysis. All analysis was done in Stata version11 (StataCorp, College Station, Texas)

Ethical Review

This study was approved by the ethics review committees of Kenya Medical Research Institute and Makerere University School of Medicine and the Institutional Review Board of JOOTRH. Since this was a retrospective study of already collected anonymous data, consent waiver was sought and obtained from the above Ethics reviews committees.

RESULTS

Baseline characteristics of study participants

A total of 1140 participants aged 15 years and above who initiated treatment between 1st January 2009 and 31st January 2011 were enrolled in this study. Of these 185 had no follow-up visit and were excluded. Subsequently 955 participants who met the inclusion criteria were enrolled; of these 66.5% were female. At cART initiation, median patient age was 31 years (inter-quartile range IQR 26-38), median CD4 counts (available for 777 patients) was 257 (IQR 164-358) and median weight 60kg (IQR 53-67); 53.1% of the patients started cART at WHO stage III/IV. A majority of the patients initiated a d4T containing first-line regimen (59.7%), as well as a nevirapine-containing regimen (89.1%) (Table 1). The baseline CD4 of 309 (39.8%) participants was collected post-CART at a median period of 2.1 months (IQR 1.2-4.1).

Table 1: Baseline characteristics of adults initiating cART at JOOTRH between January 2009 and January 2011

Variable	All (n=955)	Changed cART n=178)	Sustained cART (n=777)	Loss to follow-up (n=185)
Gender – n (%)				
Male	320 (33.5)	60 (33)	260 (33)	63 (34)
Female	635 (66.5)	118 (67)	517 (67)	122 (66)
Age median (IQR)	31 (26-38)	35 (29-43)	31 (26-38)	30 (25.5-39)
Baseline body weight (kg) median (IQR)	60 (53-67)	60 (54-67)	59 (53-67)	52 (58-68)
Baseline WHO clinical stage-n (%)				
I/II	538 (56.9)	83 (46.9)	455 (58.4)	74 (43.8)
III/IV	417 (53.1)	95 (53.1)	322 (41.6)	95 (56.2)
Baseline CD4 count (cells/µl) median (IQR)	257 (164-358)	216 (120-317)	268 (175-370)	290 (189-364)
Mising-n (%)	178 (18.6)	19 (10.6)	159 (20.5)	134 (72.4)
Stavudine				
Yes	563 (59.0)	133 (74.7)	347 (44.7)	110 (59.5)
No	392 (41.0)	45 (25.3)	430 (55.3)	75 (40.5)
Zidovudine				
Yes	248 (26.0)	29 (16.2)	219 (28.2)	38 (20.5)
No	707 (74.0)	149 (83.8)	558 (71.8)	147 (79.5)
Tenofovir				
Yes	140 (14.7)	16 (9.0)	124 (16.0)	37 (20)
No	815 (85.3)	162 (91.0)	653 (84.0)	148 (80)
Nevirapine				
Yes	850 (89.0)	158 (88.8)	692 (89.5)	149 (80.5)
No	105 (11.0)	20 (11.2)	81 (10.5)	33 (17.8)

4 participants who were included in the study were on triple NRTI (ABC, NVP, EFV), while 7 (4 in the study and 3 who were lost to follow up) were on PI based regimen.

Reasons for cART Modifications

The median follow-up time from cART initiation was 10.7 months during which a total of 178 individuals modified regimen. This represented an overall incidence rate of 18.64 per 100 person years [95% CI 16.09-21.59] over 946 person years of follow-up. The rate of modification was higher in the first year post-cART (IR; 44.08 95%CI: 36.69-52.97) compared to second (IR; 11.24 95%CI: 8.67-14.58) and the third year (IR; 3.88 95%CI: 1.85-8.12).

Table 2 shows the reasons for cART modification as reported by the clinicians. The most commonly cited reason for modification was toxicity (66.3%, IR 12.47; 95%CI: 10.41-14.94) followed by drug contraindication (12.4%, IR; 2.33 95%CI:

1.53-3.53) while treatment failure accounted for only 2.81%, (IR; 0.53 95%CI: 0.22-1.27). A further 18.5% were recorded as either others or non-adherence (2.23%). Information on adverse events was available for 34 of 118 persons who modified regimen due to toxicity. Of these d4T related peripheral neuropathy (38.2%) and lipodystrophy (26.5%) were the most common documented drug toxicities. On the other hand modification due to contraindication was mainly of NVP to EFV substitutions (68%) as a result of rifampicin-NVP contraindication with TB patients. Figure 1 further illustrates the time to cART modification; overall and stratified by key reasons for cART modification i.e. toxicity and drug contraindications. There was a steady increase in cART modification for both overall as well as by toxicity, throughout the follow-up time. The graph for toxicity closely mimicked that for overall cART modification and this was because toxicity accounted for up to 66.3% of all modifications. On the other hand the proportion of cART modification due to drug contraindication remained steadily low at less than 5% throughout the follow-up period.

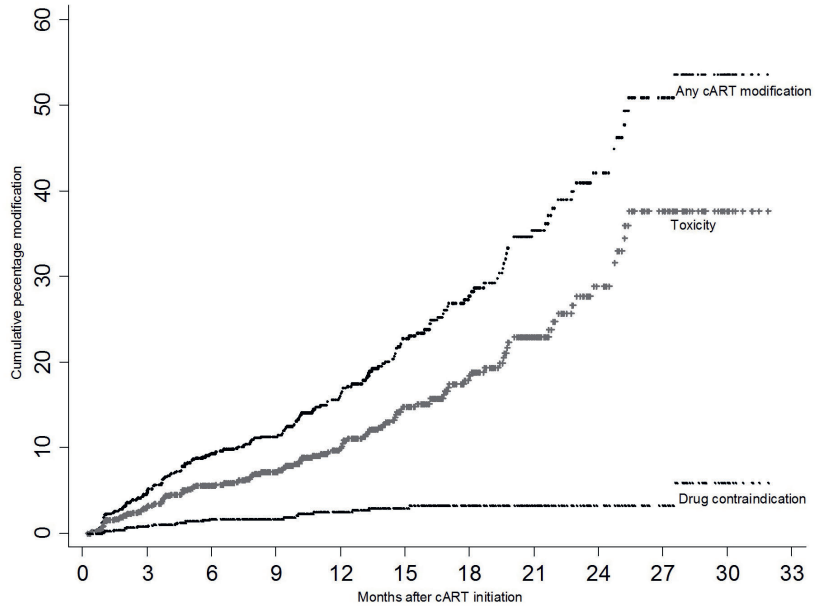

Figure 1: Kaplan Meier plots showing time to cART modification: overall and by key reasons of cART modifications.

Table 2: Reasons of first-time antiretroviral treatment modification among 955 patients initiating first-line regimen in JOOTRH in western Kenya between January 2009 and January 2011

Reason for cART modification	Overall	< 12 months	>12 months
Toxicity -n (%)	118 (66.2)	71 (62.3)	47 (73.4)
IR (95%CI)	12.47 (10.41-14.94)	27.46 (21.76-34.6)	6.84 (5.14-9.10)
Peripheral neuropathy -n	14	5	9
Lipodystrophy -n	9	2	7
Nevirapine rash -n	7	6	1
Anaemia -n	3	3	_
Hemiparesis -n	1	1	_
Contraindications -n (%)	22 (12.4)	18 (15.8)	4 (6.3)
IR (95%CI)	2.33 (1.53-3.53)	6.96 (4.39-11.05)	0.58 (0.22-1.55)
Anti-TB drugs -n	15	11	4
Treatment failure -n (%)	5 (2.81)	2 (1.8)	3 (4.7)
IR (95%CI)	0.53 (0.22-1.27)	0.77 (0.19-3.09)	0.44 (0.14-1.35)
Others n (%)	33 (18.5)	23 (20.2)	10 (15.6)
IR (95%CI)	3.49 (2.48-4.91)	8.89 (5.91-13.34)	1.45 (0.78-2.70)
Non-adherence -n	4	3	1

A majority of cART modifications were single drug substitutions (n=157, 88.2%), the drugs changed were d4T (n=92), NVP (n=48), AZT (n=9), EFV (n=9), TDF (n=2). Treatment switches from first to second-line drugs accounted for 11.8% (n=21) of all cART modifications. Overall rates for treatment modification was highest among persons initiating d4T (IR 18.83, 95%CI 15.56-22.78) based regimen as compared to either AZT (IR 4.03, 95%CI 2.17-7.49) or TDF (IR 1.43, 95%CI 0.36-5.71). This was equally the same when the rate of modifications in this NRTI's was assessed by toxicity, treatment failure, contraindication and other reasons. Between the NNRTI's the overall rate of cART modification was higher with EFV (IR 9.80, 95%CI 5.28-18.22) as compared to NVP (IR 7.17, 95%CI 5.58-9.21). The rate of modifications due to toxicity, treatment failure and drug contraindications was however higher with NVP as compared to EFV (Table 3).

Table 3: Toxicity rates of cART modification per 100 person years for individual antiretroviral regimen

NRTI	Overall	< 12 months	>12 months
d4T (%) IR (95%CI)	80 (67.8) 8.46 (6.79-10.53)	38 (51.4) 14.69 (10.7-20.2)	42 (89.4) 6.12 (4.5-8.2)
AZT (%) IR (95%CI)	5 (4.2) 0.53 (0.22-1.27)	5 (6.8) 1.9 (0.8-4.6)	0 _
TDF (%) IR (95%CI)	2 (1.7) 0.2 (0.1-0.8)	2 (2.7) 0.8 (0.2-3.1)	0 _
NNRTI			
NVP (%) IR (95%CI)	31 (26.3) 3.3 (2.3-4.7)	26 (35.1) 10.1 (6.8-14.8)	5 (10.6) 0.73 (0.30-1.7)
EFV (%) IR (95%CI)	3 (2.5) 0.32 (0.10-1.0)	3 (4.1) 1.2 (0.4-3.6)	0 -

Predictors of cART modifications

Following the identification of violation of proportionality of hazard assumption for the variables baseline weight, d4T vs AZT/TDF, AZT vs. d4T/TDF and age, a piecewise Cox-regression model was fitted for two time periods ≤12 and >12 months. This time period coincided to that which the PH assumption had been met for all the variables.

In the first 12 months post cART, baseline WHO stage (III/IV vs. I/II, aHR 1.82, 95%CI 1.25-2.66), presence of d4T in regimen (aHR 2.21, 95%CI 1.49-3.30) and a yearly increase in age (aHR 1.02, 95%CI 1.0-1.04) were significantly associated with increased risk for cART modifications. On the other hand, use of either AZT or TDF was associated with reduction in risk of cART modification (aHR 0.60 95%CI: 0.38-0.96 and aHR 0.51 95%CI: 0.29-0.91 respectively) (Table 4).

After 12 months post cART, a yearly increase in age (aHR 1.05 95%CI 1.02-1.07) baseline CD4 count ≤ 350 vs. >350 (aHR 2.45 95% CI 1.14-5.26), presence of d4T in regimen (aHR 2.75 95% CI 1.25-6.05) and baseline weight (>60kg vs. ≤ 60kg) (aHR 2.69 95% CI 1.58-4.59) were significantly associated with an increased hazard for cART modification (Table 4).

Table 4: Predictors of cART modification

	≤12 months		>12 months	
Variable	**Crude HR**	**Adjusted HR**	**Crude HR**	**Adjusted HR**
Drug related toxicity				
Age	1.04 (1.01-1.06)	**1.04 (1.01-1.06)**	1.05 (1.03-1.08)	**1.06 (1.03-1.09)**
Baseline CD4 (≤350 vs >350)	0.89 (0.48-1.66)	0.73 (0.38-1.42)	2.36 (0.98-5.70)	2.35 (0.94-5.87)
WHO clinical stage (I/II vs III/IV)	0.83 (0.28-1.38)	1.26 (0.75-2.12)	1.18 (0.65-2.12)	1.39 (0.76-2.57)
Baseline weight (≤60kg vs >60kg)			3.68 (1.89-7.16)	**4.14 (2.08-8.24)**
d4Tvs AZT/TDF	2.29 (1.33-3.96)	**2.23(1.28-3.88)**	4.32(1.34-13.95)	**4.85 (1.50-15.74)**
Contraindication				
Baseline CD4 (≤200 vs >200)	8.23 (2.48-27.31)	**5.98 (1.78-20.14)**	0.59 (0.06-5.74)	0.58 (0.06-5.68)
WHO clinical stage (I/II vs III/IV)	7.54 (2.18-26.05)	**5.92 (1.70-20.57)**		
d4T vs AZT/TDF	6.40 (1.85-22.22)	**4.10 (1.17-14.41)**		
AZT vs d4T/TDF	0.22 (0.05-0.96)	0.37 (0.08-1.62)		
TDF vs d4T/AZT			9.95 (1.40-70.75)	10.08 (1.41-72.15)

Hazard ratios and 95% confidence intervals of predictors for cART modification. Bolded values indicate independent predictors. d4T-Stavudine, TDF-Tenofovir, NVP-Nevirapine, AZT-Zidovudine, EFV-Efavirenz. *178 missing CD4 values were imputed by multiple imputation using chain equations.

Table 5: Predictors of cART modification for specific reasons

	≤12 months		>12 months	
Variable	**Crude HR**	**Adjusted HR**	**Crude HR**	**Adjusted HR**
Gender	1.10 (0.73-1.64)		1.15 (0.69-1.90)	
Age	1.02 (1.01-1.04)	**1.02 (1.00-1.04)**	1.04 (1.02-1.07)	**1.05 (1.02-1.07)**
Baseline CD4 (≤350 vs >350)	1.20 (0.71-1.99)	1.05 (0.63-1.75)	2.53 (1.19-5.35)	**2.45 (1.14-5.26)**
WHO clinical stage (I/II vs III/IV)	2.01 (1.38-2.92)	**1.82 (1.25-2.66)**	1.10(0.67-1.80)	1.20(0.72-2.01)
Baseline weight (≤60kg vs >60kg)	0.79 (0.55-1.15)		2.60 (1.55-4.37)	**2.69 (1.58-4.59)**
d4T vs AZT/TDF	2.40 (1.62-3.57)	**2.21(1.49-3.30)**	2.56 (1.17-5.61)	**2.75 (1.25-6.05)**
AZT vs d4T/TDF	0.52 (0.33-0.81)	**0.60 (0.38-0.96)**	0.40 (0.15-1.11)	0.43 (0.15-1.18)
TDF vs d4T/AZT	0.56 (0.31-1.00)	**0.51 (0.29-0.91)**	0.47 (0.15-1.50)	
NVP vs EFV	0.95 (0.54-1.66)		1.21 (0.52-2.81)	

Hazard ratios and 95% confidence intervals of predictors for cART modification due to drug related toxicities and contraindication. Bolded values indicate independent predictors. d4T-Stavudine, TDF-Tenofovir, NVP-Nevirapine, *178 missing CD4 values were imputed by multiple imputation using chain equations.

Table 5 describes the factors associated with cART modification due to drug toxicity and contraindications. Patients, who initiated a d4T containing regimen and those who were older, were significant more likely to modify regimen due to toxicity within the first year of treatment (aHR 1.93, 95% CI: 1.18-3.15 and aHR 1.03, 95% CI 1.01-1.05 respectively). After the first year of cART initiation, patients who started treatment with low CD4 counts of ≤ 350 vs. >350, (aHR 2.75, 95%CI: 1.05-7.21), a high baseline weight (>60kg vs. ≤ 60kg) (aHR 3.89, 95% CI 2.01-7.54), those with d4T in their regimen (aHR 3.84, 95% CI 1.37-10.75) and those who were older (aHR 1.06, 95%CI: 1.05-1.09) were more likely to modify regimen due to toxicity.

Similarly patients who initiated treatment at low CD4 counts of ≤ 200 vs. >200 (aHR 5.98, 95%CI: 1.78-20.14), those who had WHO clinical stage III/IV vs I/II (aHR 5.92, 1.70-20.57) those who had a d4T containing regimen (aHR 4.10, 95%CI: 1.17-14.41) were more likely to modify treatment due to drug contraindications within the first year after cART initiation. Beyond the first year of cART only patients initiating a TDF containing regimen were more likely to modify treatment due to drug contraindication (aHR 10.08, 95%CI 1.41-72.15).

Loss to follow-up and missing CD4 values

Of the 1140 participants who initiated treatment during the study period, 185 (16%) did not have any follow-up visit and thus they were probably lost to follow-up (ltfu) and were excluded in this analysis. Baseline characteristics of the participants lost to follow-up and excluded were similar to those who enrolled apart from disease stage at cART initiation, with those ltfu having advanced disease stage (p=0.003) and were also likely to have missing CD4 counts (p=0.001) (Table 1).

A further 178 (18.6%) participants had missing data on CD4 counts. These participants had similar characteristics to those whose baseline CD4 counts was available and differed only in WHO stage III/IV (57.9% vs. 40.4% p <0.001). Subsequently imputation was done for the missing CD4 values. There was no difference in the determination of predictors of cART modification when the analysis was done without the imputation, with only slight adjustments in the hazard ratios (i.e. at time periods >12 months, age (aHR 1.04 95%CI 1.02-1.07), CD4 counts ≤ 350 vs. >350 (aHR 2.64, 95%CI: 1.25-5.59), d4T (aHR 2.52 95% CI 1.14-5.55 and baseline weight (>60kg vs. ≤ 60kg) (aHR 2.23 95% CI 1.31-3.79).

However there were differences in the predictors of cART modification due to toxicity and contraindications when analysis was done without imputation of missing CD4 counts. Low CD4 counts ≤ 350 vs. >350 (aHR 2.87 95%CI 1.11-7.42) was a significant predictor of cART modification due to toxicity at >12 months, in addition to age, d4T and high baseline weight. On the other hand, low CD4 counts ≤ 200 vs. >200 was no longer associated with cART modifications due to contraindication at ≤ 12 months post treatment initiation.

DISCUSSION

We observed a moderate incidence of treatment modification; 18.64 per 100 person years within a median follow-up period of 10.7 months in this adult cohort of patients who started cART as part of routine clinical care in a resource limited setting.

The relatively moderate rates of cART modifications are synonymous with those reported from similar settings [11, 14, 23], but are slightly higher than those observed in programs and clinical trials [14, 24]. This difference is likely due to close treatment monitoring or potential selection bias of persons enrolled in clinical trials and programs as compared to those in routine clinical settings. The rates are however still lower than those observed in developed nations where cART modifications are as high as >50% [9, 25, 26]. The difference may probably be due to limited cART options or the pre-determined population based ART guidelines in these settings, which is likely to influence the clinicians' decision on cART modification.

Toxicity was the most common reason for cART modification similar to what has been reported in other studies [8, 10-14, 27]. Stavudine accounted for majority of toxicity related cART modification with risk increasing with time on treatment. Previous studies have shown a high toxicity profile for d4T-based regimen presenting mainly as acute lactic acidosis and long term mitochondrial toxicities (lipoastrophy and peripheral neuropathy)[28-32]. This has consequently led to the current WHO guidelines recommending d4T phase-out and the subsequent adoption of TDF or AZT drugs which have better tolerability [6]. While d4T use in affluent nations has subsequently declined, African countries still rely on d4T-based regimen due to high cost of TDF [33, 34]. However cost-effectiveness analysis comparing high cost TDF to d4T showed a general preference for TDF but

with relatively high cost of approximately 17 US$ per QALY increase per month [33]. In this study the presence of TDF in the first-line regimen was observed to have a 49% reduction in the risk of cART modification; an indication of its good safety profile as has been reported in other studies. Contrary however to findings from some studies was the absence of AZT risk for cART modification. In our study, patients on AZT had a 40% reduced risk, which may either imply that AZT equally had a good toxicity profile in this population or may portray the resistance by clinicians for AZT-based modifications, which may appear milder than those for d4T. Apart from the NRTI drugs, both EFV and NVP had a moderate rate of toxicity related modifications with higher rates in the first year post-cART. This is in concordance with the reported occurrence of Nevirapine (rash and hepatotoxicity) and EFV (central nervous toxicity) adverse events usually occurring at early stages of cART initiation [6].

Modifications due to drug contraindications were also significant with changes due to TB treatment accounting for the majority. This reflects the high level of TB burden in this region and the need for focused TB prevention and screening programs among HIV patients on care and treatment. Both d4T and TDF were significantly associated with risk of cART modification due to drug contraindication. This may probably be due to the reported increased risk for peripheral neuropathy when both Isoniazid TB drugs are used together with d4T [35]. On the other hand, TDF association with cART modification experienced only after one year post-cART, could have been confounded by the relatively few drug contraindication related modifications experienced after one year of treatment. However some studies have also reported potential risk of increased nephrotoxicity when TDF is used with some TB drugs (rifampicin, streptomycin and pyrazinamide) and this may probably influence TDF modification in TB patients [36].

Treatment switches due to ART failure were low at less than 1% in the studied population, which may suggest a high efficacy of first-line drugs in this region or a shorter follow-up period or the lack of proper mechanisms to identify treatment failure in such settings. Due to lack of adequate viral load and drug resistance capacity in resource-limited settings, CD4 values and clinical assessment are usually used to assess treatment failure. However previous studies have shown a poor correlation of CD4 and clinical assessment with treatment failure, leading to late detection of treatment failure and subsequent late switches [37, 38].

Following this, the revised WHO guidelines now recommend the use of routine viral load as a better monitoring strategy in determining treatment response [39].

However about 2.2% of the study participants were on second-line regimen at the end of the study. This could imply that although toxicity may have been the main reason for treatment modification, it is likely that this may have been accompanied by treatment failure necessitating switch of regimen rather than single drug substitutions. This further corroborates existing evidence for toxicity mediated treatment failure through non-adherence and further calls for close monitoring of patients on treatment to prevent loss of salvageable regimen through avoidable switches.

Increase in age at cART initiation was found to have a moderate risk for modification similar to what has been observed in other studies. Baseline weight was also a significant risk factor for treatment modification, in which patients weighing over 60kg were twice at risk for modification. This is synonymous to what has been observed in other studies showing the association between NVP and d4T based toxicities and higher baseline body weight [40, 41]. This could also explain the observed greater than four times risk of treatment modification due to toxicity for heavier persons after 12 months of treatment.

The risk of cART changes also increased with the stage of the disease as reflected in both CD4 counts and WHO disease staging. These findings are synonymous with what has been previously reported showing that sicker patients are more likely to modify regimen due to a higher risk of adverse events [42-44] . In addition sicker patients are also likely to be on other medications for opportunistic infections and may equally be at risk of changing treatment due to drug contraindications. These findings further build up on the evidence that treatment initiation at higher CD4 counts and at lower WHO disease stage leads to increased patient's durability on the initial first-line regimen. In Kenya, the level of HIV status awareness is still low and majority of patients are likely to know their status mainly when they are at advanced stages of the disease [45]. This may result in poor treatment outcomes as well as increased risk of cART modifications. The current push for more aggressive HIV testing programs like provider initiated counselling and testing (PITC) and home based care and testing (HBCT) in addition to the routine voluntary counselling and testing (VCT) are likely to improve this situation, by timely placement of patients on treatment and this could subsequently reduce the risk for cART modifications.

Our study has limitations. First, being a retrospective analysis of records, various errors experienced with such a study design are likely to be present. This includes the potential for random misclassification error during clinician recording. In addition, non-specific clinician's recording of the reasons for cART modification in some patients was non-informative as it was only recorded as "others'. There was also the potential for selection bias as about 16% of the patients were lost to follow-up. It is likely that the reasons leading to the loss to follow up may have been linked to the outcome of the study in that some of this patients may have opted out on treatment due to adverse events experienced, and this could have the potential of under-estimating the magnitude of cART modification. Moreover baseline CD4 values for some participants were collected within four months after treatment initiation. Although this may reflect delays in results relay, it could also bias the results, if the CD4 were actually determined after treatment initiation, since some patients are likely to respond quite well after treatment leading to significant difference in the baseline and 4 months post-cART CD4. Finally the study findings are limited to settings where similar regimens are in use, as in our study majority of the patients were on NVP and d4T based regimen.

Notwithstanding the limitations, this study provides unique findings with regard to incidence and predictors of cART modifications and had several strengths. First, this study was carried out in a routine clinical set-up, whose characteristics may represent the routine standard of care in most resource limited settings and thus allowing generalizability. Secondly, our study assessed the rate of cART modification at two different time periods; during the first year and after the first year post-cART initiation and provided information on associated factors for treatment modification at the two time periods as well as for major specific reasons of cART modification. This is vital in informing clinicians on the time at which patients are at risk of modifying treatment and the possible factors that could influence modification at those time periods.

In conclusion, we report a moderate rate of cART modification from a routine clinical set-up in western Kenya. Toxicity was identified as the most common reason for cART modification while factors predictive of the change were advanced WHO staging, low CD4 counts, a yearly increase in age, a higher baseline weight and the presence of d4T in regimen. On the other hand, the presence of zidovudine and tenofovir in regimen led to a reduction in the hazard for modifications.

The findings of this study have several implications for the management of patients on treatment. First, the identification of toxicity as the main reason for cART modifications calls for the need for early and proactive management of toxicity in order to prevent poor treatment outcomes including treatment failure. Second, the identification of low CD4 and advanced disease stages as important predictors for cART changes indicates that adoption of revised early treatment initiation strategies is likely to be beneficial in the prevention of cART modification. Finally, the continuous identification of d4T as an important predictor of cART modification calls for an accelerated implementation of the WHO guidelines recommending d4T phase-off in favor of TDF/AZT based regimen in resource limited settings as these is likely to significantly minimize treatment modifications.

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Chapter 5

Long-term antiretroviral treatment adherence in HIV-infected adolescents and adults in Uganda: A qualitative study

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ABSTRACT

Background

Long-term success of HIV antiretroviral therapy requires near-perfect adherence, maintained throughout one's lifetime. However, perceptions towards ART and patterns of adherence may change during the life course. We assessed challenges to long-term adherence in adolescents and adults in three regional HIV treatment centers in Uganda.

Methods

We conducted 24 in-depth interviews and 2 focus group discussions with a total of 33 health-care providers and expert clients (HIV patients on long-term ART who assist with adherence support of fellow patients). Interview topics included experiences with patients on long-term treatment with either declining adherence or persistent poor adherence. Transcribed texts were coded and analyzed based on the social-ecological framework highlighting differences and commonalities between adolescents and adults.

Results

The overarching themes in adolescents were unstructured treatment holidays, delays in disclosure of HIV status by caretakers, stigma, which was mainly experienced in boarding schools, and diminishing or lack of clinical support. In particular, there was minimal support for early and gradual disclosure for caretakers to the infected children, diminishing clinical support for young adults during transition to adult-based care and declining peer-to-peer support group activities. The predominating theme in adults was challenges with treatment access among temporary economic migrants. Common themes to adults and adolescents were challenges with disclosure in intimate relationships, treatment related factors including side effects, supply of single tablets in place of fixed-dose combined drugs, supply of drug brands with unfavorable taste and missed opportunities for counseling due to shortage of staff.

Conclusion

Adherence counseling and support should be adapted differently for adolescents and adults and to the emerging life course challenges in long-term treated patients. Programs should also address constraints experienced by temporary economic migrants to ensure continuity of treatment within the host country.

INTRODUCTION

Unprecedented scale-up of antiretroviral treatment (ART) in sub-Saharan Africa has remarkably changed the face of HIV epidemic from the previous fatal illness to a life-long chronic disease [1]. Subsequent sustenance of long-term and successful treatment outcomes depends primarily on achieving sustained viral suppression, supported by optimum life-long adherence [2]. Remarkably high levels of adherence have been reported in sub-Saharan Africa in comparison to western countries [3], but these findings contrast with those from other studies reporting increasing levels of patients with treatment failure [4,5], low retention [6] and high mortality rates [7]. Fewer studies have assessed adherence in long-term treated patients, despite the growing numbers as ART programs mature in the region [8–10].

Adherence over time may decline with duration of treatment [11,12] improve [8] or stabilize [13]. The reasons for decline in long-term treated patients may vary from those at the initial treatment phase [10,14]. As patient's health improve, change in perception towards the disease, laxity to medication restrictions such as alcohol and tobacco use, non-disclosures in new sexual relationships and other life-course events may influence adherence to medication [10,14].

Of particular importance to long-term adherence is the emerging cohort of perinatally HIV infected children who are increasingly surviving into adolescence and adulthood. In 2014, about 2 million adolescents (aged 10-19) were living with HIV-AIDS (ALWHA), with 1.2 million (61%) in Eastern and Southern Africa [15]. ART adherence in ALWHA in sub-Saharan Africa is reported to be lower as compared to other age groups [16–18] including children [17,18] and declines also with increasing age [16,18]. Subsequently, adolescents have comparatively poor treatment responses [16,19] including high mortality [15,16].

Few studies have assessed long-term adherence among adolescents and young adults in sub-Saharan Africa [16,17,20–23]. A recent study in Uganda reported stigma, discrimination, attending rural health facilities and disclosure issues as main barriers to adherence [20]. Peer support groups, counseling, supportive health care workers, short waiting time, provision of food and transport were facilitators. More contextual data is needed to guide sustenance of long-term treatment adherence in this group. Although health-care workers play a pivotal

role in providing adherence support and are thus usually more informed through their interactions with the patients and their understanding of the health-care system, few studies have included them when assessing adherence.

To conceptualize the barriers and facilitators to long-term ART adherence in both adolescents and adults, we conducted a qualitative study with health care workers and expert clients from three regional referral HIV treatment centers in Uganda.

METHODS

Theoretical approach

This descriptive qualitative study draws from the social-ecological model (SEM) framework, which considers an individual's behavior in this case 'ART adherence' as being a result of dynamic and complex interactions of factors at the various social-ecological levels in which the individual is situated [24]. An individual's behavior is hence shaped based on the information, influence and interactions he/she obtains within one's social-networks, social environment and institutions.

In sub-Saharan Africa, this includes cultural aspects, religious and society beliefs on the cause of the disease and its management [25]. In this way the construes of adherence do not only include the "how to adhere" as guided by the medication aspects and the "why to adhere" based on individual aspects but also the social component which influences the willingness and ability to adhere[25]. Indeed, whether a person will adhere to treatment in such settings is heavily grounded in the social context and an individual must not only negotiate the how's and why's of adherence but also the societal positive and negative influences.

Moreover the individual is also dependent on the resources available to assist him/her to access and adhere to treatment [25–27]. In this way adherence to medication does not simply depends on the individual's behavior but also upon structural factors within the individual social and environmental context.

In addition to social and structural factors, a patient's adherence behavior is also influenced by program level factors. This includes such aspects as availability of drugs, distance and transport costs to ART clinics, quality of care, relationship with caregiver, as well as treatment factors such as dosing complexities[25–27].

Setting

The study was conducted in three regional referral centers (RRCs) in Uganda; the Joint Clinical Research Center (JCRC) in Kampala and the RRCs in Fort Portal and Mbale, both run by the MOH. JCRC Kampala provides care to approximately 15,000 clients, 2,000 of whom are HIV-infected children and adolescents. RRC Mbale has approximately 4,200 patients on ART, with 400 children and adolescents; and RRC Fort Portal has approximately 7,474 patients, with approximately 500 children and adolescents. Ethical approval for this study was received from the Hospital Clinic-University of Barcelona Ethical Committee Board, the Joint Clinical Research Center ethical review board and the Uganda National Council for Science and Technology.

Data collection

Data collection took place from May to August 2015, using semi-structured interviews (n=24) with open-ended probes and focused group discussion (n=2). We recruited participants based on purposive sampling so as to include more informative persons with regard to long-term adherence. In particular we included health-care workers who had longer years of experience with patients (Table 1), those specifically handling adolescents and adolescents transitioning to adult-based care (transitioners). We also included expert clients including adolescents themselves. Expert clients are HIV+ patients on long-term ART who assist the health-care workers mainly with adherence support of fellow patients. They play a vital role in bridging the gap caused by a limited health-care workforce but more important are able to reach out to patients at a more personal level based on their own experiences with the disease and treatment [28,29]. They provide counseling, motivate fellow patients by sharing their own personal experiences, conduct active tracing of patients lost-to-follow-up, offer adherence support and sometimes also assist health-care workers in minor clinical work such as in triage, patient flow and translation [28,29].

Data collection guidelines were designed to elicit health-care providers' and expert clients' views on declining adherence after long-term treatment and persistent poor adherence. Specifically, the interview guides were set to elicit personal experiences of the health-care workers with poor-adherent patients and included the reasons given for the non-adherence as well as the management of these patients. The guides were also developed in such a way as to elicit broader aspects within the social-ecological framework, including personal-related

barriers, social, and institutional-related barriers. In this way, the interviews provided deeper insights of both health-care workers personal experience with the clients as well as the broader systemic challenges known to impact on adherence. The guides were also adapted to gather the personal experiences of expert patients with ART adherence.

Through the study the guides were also progressively adapted within a *cyclic research design* where data gathered and analysed informed the next interviews. The iterative process was continued until saturation. Interviews were conducted at the clinic setting and lasted on average for 60-120 minutes. All the interviews were conducted in English by SCI; a junior research scientist-*MSc*, with close support from MR; a senior social scientist-*PhD*. All participants were provided with a detailed description of the study including the consent process and verbal consent was obtained and tape-recorded before each interview took place. The use of verbal consent was approved by the JCRC ethical review committee and was based on minimal risks associated with the study. All interviews were transcribed verbatim. Random fragments were sampled for quality checks and the data was imported and analyzed using the qualitative software package NVivo v.10.0 (QSR International). We employed a framework approach to analyze the data. We specifically used the social-ecological framework to create an initial coding guide that was also designed to highlight differences between adolescents and adults. This was done through discussions between SCI and MR. SCI then coded all the transcripts while MR checked the coded outputs. Codes were then revised and refined based on relevant and recurrent themes emerging from the data. Relationship codes were then created to analyze linkages and memos employed to document emerging themes. We then developed matrices to organize and iteratively compare the data indicating differences and commonalities between adolescents and adults.

Table 1: Characteristics of study participants

Total participants	In-depth interview -N	FGDs-N
Doctors	5	2
Nurses	11	6
Pharmacists	3	2
Counselors	9	6
Expert clients	5	1
Characteristics	N	
Years of experience with ART care and treatment (Median, IQR)	7 (2-23)	7 (4-8)
Female/male gender	22/11	14/3
Site	N	N
Joint clinical research center,	23	8
Mbale regional referral center	7	_
Fort Portal regional referral center	3	7

RESULTS

A total of 33 individuals participated in this study including eleven nurses, nine adherence counselors, five medical doctors, five expert clients and three pharmacists (Table 1). Of these 24 participated in the interviews and 17 in the focus group discussions.

Eight key themes regarding long-term adherence were identified from the data, four of which were predominant among the adolescents; unstructured treatment holidays, delays in disclosure of HIV status to perinatally infected adolescents, stigma in boarding schools, and diminishing or lack of family and clinic support (extent that the clinic staff/structure helps the patient to adhere). In adults, challenge with treatment access among temporary economic migrants was the predominating theme. Common themes to adolescents and adults included disclosure in intimate relationships, treatment related factors and missed opportunities for counseling due to shortage of staff.

Barriers specific to adolescents

Individual-level factors

Unstructured treatment holidays were noted amongst adolescents and were expressed as a desire to experience a drug-free life or a quest to understand the effects of being off drugs. However, this was also as a result of underlying factors

that included drug fatigue, pill burden, depression and stigma. Addressing these underlying factors could lead to resumption of treatment although in some cases the patients resumed treatment due to deteriorating health.

> *"There's a girl who...has been on treatment for some time but her viral load was high, so when I tried talking to her she was like "ah aah [No!]! Don't tell me anything, I know everything, I've decided I'm not taking [the drugs]".By the way she came today I was surprised! ...when they asked her about her drugs she was like "I felt like I was tired of the drugs, so I needed a break". Sometimes adolescents they give themselves breaks...for like 3 months but when he becomes sick, very sick, he comes back and says, "Now I want medicine". It's like they want to first see what will happen when [on] a break".* ***(Expert client-1)***

Social-level factors

Delays in disclosing HIV status to perinatally infected children prior to adolescence were common and could lead to non-adherence. In accordance with government regulation [30],it is the duty of the caretakers to disclose to their children, but they were reluctant and cited barriers such as fear of being blamed for the infection, fear of unintended disclosure of the parent HIV status by the child, fear of anticipated negative reactions and concern for lack of cognitive ability of the child to comprehend the implications of the disease on their health. Due to this, caretakers would sometimes lie to their children about their condition indicating that they were suffering from other chronic diseases like asthma or cancer. The concealment of status by implicating other diseases could however impact on the adherence of the adolescents as it foils the understanding of the importance of adherence and subsequent consequences, which are specific to HIV treatment. Moreover, this could result in anger and depression when the adolescents become aware of their condition. Health-care providers could at times be obligated to assist with disclosure mainly when it was imperative for the child to know their status for example during adherence support at treatment failure or treatment switch which was done as a means of averting further failures.

> *"This time we had a very strict doctor whom they found [during clinic visit]... she said, "You must disclose…because the boy is now 15 years and he is already on second-line [treatment]…So, the doctor took her [guardian] to the counselor by force, and they made her to disclose"* ***(Pharmacist-1)***

Participants also noted that delays in disclosure were partly due to lack of supportive mechanisms to assist caretakers with disclosure.

> *"Caretakers can manage when they are prepared on time about disclosure. Usually they are stuck when you tell them [abruptly] that they need to tell the child. They are not prepared on how [to go about it]?"* ***(Counselor-3)***

It was also noted that the lack of support for disclosure could result with poor or partial disclosures where children knew about their HIV status but did not fully comprehend the implications of the disease. Late or poorly disclosed adolescents were likely to stop treatment, react negatively to their caretakers and sometimes deny their HIV status.

Diminishing or lack of family support: Adolescents living with biological parents were perceived to have better adherence compared to those living with caretakers. The latter were reported to lack support for food, transport, medication reminders, and accompaniment by caretakers during clinic visits. Some adolescents also reported that the caretakers denied them education support on the misconception that they would die soon. However, as the adolescents grow older the need to be independent as well as other structural barriers could result with diminishing influence of the parents on the adolescent's adherence behaviour or support.

> *"I'm asking [the mother] about so and so, she says, "his drugs are at home" So the boy would take [a few] tin[s], if he is dispensed with 3-months' supply on 2nd line, he would take 2 tins, leave the 4 at home. Then the mother would not bother, how comes, its long since he left his medicine. You would call her and she says, "so and so took medicine, some is still at home". Till one time I told the woman that you carry all whatever you have at home [and bring to the clinic], she carried tins and tins and returned them"* ***(Counselor- 1)***

Perceived and experienced stigma in boarding schools: Adherence among students in boarding schools was reported to be poor with the majority already being on second-line treatment. Poor adherence was linked to stigma, and this was influenced by where and how the students take their medication. Two groups emerged in the discussion: students who keep their own medication in the dormitories and those who keep them with the school nurse. Keeping drugs with the school nurse was reported to be beneficial due to likely reminders, close

monitoring and easiness in getting permission for clinic visits. The preference to remain with the drugs was however due to having not disclosed to the school administration, the desire to be independent and fear of involuntary disclosure by the teachers or nurse to fellow students. Moreover, prevention messages such as 'AIDS Kills' placed in most schools also inadvertently stigmatized the infected students making it difficult for them to disclose.

Students keeping their own medication were said to face lack of privacy, anticipated (perceived) and enacted (experienced) stigma from peers and they also sometimes failed to access medication from the dormitories on time.

> *"There is a girl we lost, she passed away, she was 18... she had [experienced] stigma at school because they came across her drugs in her suitcase, and they pulled them out and they put them there and put her [medical] card on her bed and she was a head-girl and that killed her [spirit]! She had to switch school. Most of them you get these calls, when they are saying they have found out, you see, so she had to switch out schools"* ***(Counselor-3)***

However, those keeping their medication with the school nurse also reported facing stigma emerging from their peers who persistently inquire of their frequent visits to the infirmary. Moreover these students could also experience stigma arising from inadvertent disclosures by the school nurse or administration.

> *"In the first school I disclosed.... but our nurse discriminated me...whenever the time of [taking] drugs approached she could come in the class [and say], "you don't know that you have to take your drugs? Come and take your drugs" while all other students are there, [listening]… So they [other students] used to inquire what drugs do you take, the nurse is [always asking]…"you know you have your problem come and take your drugs ah"...If you don't want you will die" ah, while other people are listening so [you get such] nicknames, "Mr. drugs, that is madam drugs"* ***(Expert client-2)***

Health-care level factors

Declining or lack of clinic support: Most ALWHA at the study facilities were reported to have been infected through vertical transmission and had started ART early in life at the pediatric clinic. At 18 years of age they are expected to transition to the adult-based care. Transitioning was however a challenge due to difficulties in integrating with adult patients, reduced attention from health-care

providers, long waiting hours, lack of medicine for treatment of other ailments and a general sense of abandonment from health care workers. Subsequently some of the adolescents would be lost-to-follow up.

> *"When we would transit them to adult [clinics], some of them would fail to come to pick their drugs, they were not getting the attention they were getting in the pediatric [section], because in pediatric, they are few [in numbers] and we try to know them personally"* ***(Pharmacist-1)***

Although most participants expressed the importance of peer group support across all patients, it was noted that a decline in funding had either resulted with fewer activities or the complete phasing-out of these groups. The peer groups were reported to have served a pivotal role in providing an avenue for strengthening adherence, dealing with emerging challenges, providing motivation from peers and HIV-infected role models. Moreover, they also served as platform for social networking, where participants would get potential life partners. The subsequent decline of these groups was reported to be have impacted especially the adolescents with some of them opting out of care.

> *"Other challenges are the lack of peer groups and activities for adolescents. We used to have them but because of funding they stopped. And some adolescents dropped off after these activities and some even died. They had a strong belonging to these groups"* ***(Counselor-6)***

Moreover, while it was noted that adolescents were a group in need of more counseling, this was hindered by a shortage of counselors. Frequent transfers of health-care providers due to shortage in staff also posed a problem to the adolescents who found it a challenge to confide and discuss freely about their health with new health-care providers. The use of social media coordinated by the clinic was a notably noble alternative, which was used both socially and formally to facilitate health-care providers and peer-peer counseling but was said to lack sufficient participation from health-care providers.

> "*And another thing is all about our social network pages, especially the Facebook ones. We need more counselors there...one can post via a friend so that others can know how to handle the problem but you fail to get even a counselor who can comment on the post or give advice on the post"* ***(Expert client-2)***

Barriers specific to adults

Individual-level factors

Temporary migrants and challenges with treatment access: Mobile persons especially those travelling temporarily to nearby countries for business comprised a substantial majority of patients and they had notably poor appointment keeping and drug pickups. Reasons given included lack of funds to cover for transport costs, perceived feeling of wellness hence prioritizing business to drug refill visits, desire to match clinic visits with trips for replenishing business stocks, and political conflicts in the host countries (mainly Southern Sudan and Congo), which affected their travel.

> *"I've had experiences of people saying that they travelled to Juba [South Sudan]...they go there for business…hoping to come back like after 3 months and the majority of them were complaining of this recent war that was in Juba, they could not make it…others talk about transport, they didn't get enough money to come back [for drug refills] … then others talk of no proper health facility elsewhere that could give them drugs, otherwise they would have continued taking HAART…others would point-blank tell you they were not feeling ill, health-wise they were feeling ok, and since they were doing their business they decided to continue [with] their business".* ***(Counselor-4)***

Other mobile populations included commercial sex workers, truck drivers, persons relocating to the villages for farming, and temporary emigrants. As a way of supporting these patients, they were supplied with drugs for 6-12 months instead of the custom 3 months so as to minimize on the number of drug-refill visits. Relatives or friends could also pick up drugs periodically on behalf of the patients but they would still be required to visit the clinics for blood tests at least once or twice a year. Despite these interventions, some clients still had poor appointment keeping prompting the health-care providers to encourage them to transfer to near-by facilities. Some were however reluctant to transfer-out citing poor standards in other facilities. Some migrants also reported reluctance by some of the host countries to provide them with ART.

Common barriers

Individual-level factors

Disclosure in intimate relationships: New challenges with disclosure were cited when engaging in romantic relationships leading to compromises in adherence and potentially fuelling new infections. In adults, non-disclosure was linked to avoiding marital conflicts, fears of losing the partner or/and financial support. Adolescents generally lacked the skills to disclose and also feared losing their partner.

> *"In young adults, there are those getting boyfriends and girlfriends. They don't disclose, and that is what prevents them from taking the drugs. They feel ashamed and don't want to lose their partners"* ***(Expert client-3)***

While support on disclosure was offered at the clinic, some health-care providers acknowledged their own bias when counseling discordant couples because they did not approve such relationships. This in turn could lead to the clients not to seek for disclosure support in subsequent times.

> *"They will tell you, when we take these partners to the hospital.... they are quick to judge you, you who is positive and yet you have brought in your partner really for testing. So, they don't help them and they end up breaking up instead. They don't support them into this relationship and guide them on what to do. Instead they say heey! Trouble! So, they separate them. So [they] are stuck with their relationships, they can't come out open, [they have questions] who is going to support [me]... where should I go for counseling anyway? Because we are already biased"* ***(Counselor-03)***

Health-care level factors

Treatment-related factors: Treatment side effects were also a concern for long-term treated patients in particular those arising after switch to second-line regimens. In adolescents this was said to result with re-emergence of stigma due to changes in body fat distribution from lopinavir-based treatment, and yellow eyes (jaundice) from atazanavir.

"We have many people who were changed to second-line, they were fat but now they are small. And they ask the doctor's why? Does 'lopinavir' takes off the kilograms...they fail to adhere whenever they see they have lost weight, they are looking bad, it seems as if 'lopinavir' is the cause" ***(Expert client-2)***

There were also challenges with drug supply logistics where separate pills were supplied for use by patients on fixed dose combinations (FDC). Moreover, there were reports where facilities were supplied with pediatric formulations involving multiple low milligram tablets, for use by adults thereby increasing the pill burden.

"They send pediatric drugs for use in managing adults. So, what we do, if the person has been taking like one pill, she will take 3 or 4, to make an adult dose. It is a challenge, which increases on client's pill burden" ***(Counselor-8)***

Moreover, adults also expressed a dislike of the sweet taste of pediatric drugs. In addition, facilities reported being supplied with drug of different brands, some that had bitter taste (uncoated drugs) and sometimes with varying colors and sizes. This was reported to confuse the patients and affect their adherence especially when they were given the bitter drugs. There were also claims of emerging side effects associated with change of drug brands.

"When we change from one company to another, some do experience some abnormal side-effects and they always say: "when I was taking the other type I didn't have any problem but this time...". Before we used to say that maybe they are used to this type of drug, but it does really affect them. When you change them and put them to a previous one, the complaints do go away." ***(Pharmacist-2)***

Although there were no cases of drug stock-outs, there were reports of insufficient supply of drugs or those with shorter expiration period, which necessitated more frequent drug refill visits. There was also a limited supply of third-line drugs, which was mainly available to patients enrolled in medical research.

Staff shortages and missed counseling opportunities: Staff shortage leading to increased workload and long waiting times resulted in missed opportunities for adequately counseling the patients on vital information such as interpretation of laboratory test results. This could lead to misconceptions with some patients inferring that an undetectable viral load implied that they had been cured of the disease.

"So when [viral-load test] results were undetected, the person [clinician] would say that 'akauka takalabika' directly translating into the virus is invisible, so the patients would take it as gospel truth [or]...they say 'akauka tekalio', meaning the virus is not there.... The health worker should explain to those patients: last time we tested your viral load is undetectable, it doesn't mean you are healed, it simply means that you are taking your drugs well and the virus has just slept so you have to keep taking drugs so that the virus keeps sleeping. When you stop, it wakes up. But most of them don't get that explanation". ***(FGD-2)***

DISCUSSION

Our study highlights pertinent insights into long-term ART adherence further distinguishing between adults and perinatally infected adolescents. In adults, a return to health with resumption of social and economic activities resulted in new dilemmas, potentially affecting their adherence. In adolescents, the challenges in adherence were mainly related to stigma, disclosure and declining clinic support.

Mobility and adherence in adults

Emerging challenges to adherence with treatment progression in adults identified in previous studies suggest differences between early and long-term treated patients [10,14,31–33]. These studies have shown changes in disease perceptions, non-disclosure in intimate relationships, poor adherence to counselors' instructions and re-emerging and persisting stigma as key challenges [10,14,31,33]. In our study, we further highlight challenges associated with mobile groups especially temporary business migrants as they strive for social and economic improvement. Although studies have documented the challenges faced by long-term migrants in accessing HIV care [34], there is still limited information on circular migrants, defined originally by Zelinsky as short-term, repetitive, or cyclical [35], which encompasses the temporary business migrants. The initial impediments to engagement in care and early adherence such as lack of transport and proper health care services [26,27] re-emerged in these long-term treated migrants, and were linked to a reluctance to transfer-out from their home facilities. In our study sites, preferential supply of treatment for longer duration as well as allowing treatment partners to pick-up drugs, were strategies that helped to maintain these patients on treatment. The preference to further transfer patients to closer facilities was however a challenge citing the

reluctance by the host country to provide treatment to migrants. This is contrary to the recommendations by the 'Global commission of HIV and the law', directing that migrants be accorded equal treatment as citizens in matters relating to HIV[36].

Stigma and ALWHA

As with previous studies, stigma was reported to impact on ART adherence especially among adolescents in boarding schools [20,23,37,38]. These findings corroborate other studies reporting the challenges faced by ALWHA in schools including discrimination from their peers and educators suggesting an increasing need for sensitization and training on their rights and special needs in school settings [23,38]. In addition, there is a need for a balanced approach in the design of HIV prevention messages in schools as the current ones such as 'AIDS kills' are discriminatory and further prevents ALWHA from disclosing and seeking support from either their peers or the school authorities.

Stigma among ALWHA was also associated with treatment side effects in particular visible body changes such as lipodystrophy [39] and 'yellow eyes' [40] from atazanavir. As adolescents are in particular careful about their physiques, it would be vital for treatment programs to consider implementing a personalized treatment management approach including switching to less toxic regimens once the patients have achieved viral suppression [41].

Lack or declining clinic support for ALWHA

The pivotal role of health-care providers and the clinic environment to ALWHA is well known, with various studies showing the need for supportive early disclosure to caretakers [42–44], provision of adolescent friendly services [45], role of peer groups [20,45,46] and support for gradual transition to adult based care [45,46]. Despite this information, lack of clear guidelines and support was associated to late, incomplete and poor disclosures by caretakers and the subsequent decline in adherence [42]. Moreover, the need for gradual transition to adult-based care was a pertinent issue among adolescents in these facilities. Although guidance for transition exist in developed countries, there is little support for adaptation and implementation in sub-Saharan African settings [47]. Moreover the decline in funds leading to the phase-out of peer-peer support and the lack of adolescent-friendly counselors impacts negatively among the adolescents whose identity is strongly molded through the interactions within the clinic environment [48]. It is worth noting that most vertically infected adolescents are also orphaned and

depend more on the close ties formed at the clinic for support and empowerment against the various challenges inherent in the community [23]. These findings continue to demonstrate the need for increased support of this group in the wake of increasing deaths of ALWHA in sub-Saharan Africa [16]. Alternative use of social media was seen as a platform to support adherence and cohesion among ALWHA. However, formal integration into existing health systems, coupled with sensitization and training of health-workers is needed to promote the use of these potential m-health tools in fostering adherence counseling in these settings.

Challenges with drug supply logistics and influence on adherence

Apart from counseling, erratic drug supply logistics were also reported in these settings and in particular influenced adherence among patients on long-term treatment. This suggests the need for instituting quality systems to ensure continuous supply of appropriate regimen and formulations, prevent supply of uncoated bitter pills, and ensure sufficient communication to patients concerning the drug characteristics whenever there is a change in brand.

Study limitations

There are some study limitations. First, we mainly included the views and experiences of health-care workers and expert clients and we may have missed other pertinent information from patients, including adolescents. The study had been specifically designed to address the barriers of long-term adherence based on the experience of health-care workers. The inclusion of health-care providers was based on the premise of the in-depth information they possess based on the nature of their interaction with HIV patients [49] and the additional advantage in their understanding of the health-care system. Our further inclusion of expert clients including adolescents provided crucial information to the study because of their a) Interactions with the patients at a more personal level: because of their own personal experiences with the disease and treatment, expert patients are viewed to be more relatable and empathetic to the challenges experience by the patients. This was vital among the adolescents who sometimes perceive the adult clinicians as being insensitive to their needs.

The expert clients also have closer relationship with the patients, sometimes even visiting them at their homes to help address some of the difficulties they face. b) their own personal experience: In addition to the experience they have with the patients, the expert clients also shared their own experiences on the challenges they face with long-term ART adherence c) their view from both

the perspective of the patient as well as from the health-care perspective: The information provided by the expert clients also reflects a balanced view of the barriers related to the health-care system owing to the fact that they serve as health-care workers and are also patients in the same facilities.

Second, our study was carried out in regional facilities and may underestimate the broader view especially from lower-level health facilities.

CONCLUSION

Successful adherence among long-term treated patients in these setting is hampered by life-course events and is especially challenging among adolescents. There is thus need for programs to tailor adherence interventions to the emerging needs including support for disclosure to intimate partners and ways of ensuring continuity of treatment among temporary economic migrants. Moreover, programs should endeavor to support ALWHA in particular by offering support to caretakers for early and gradual disclosure of HIV status, supportive gradual transition to adult-based care as well as maintaining functional peer-support groups. Lastly there is need to train and sensitize educators and students against discrimination of ALWHA as well as empowering the infected children to cope with stigma in boarding schools.

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Chapter 6

Emergence of untreatable, multidrug-resistant HIV-1 in patients failing second-line therapy in Kenya

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ABSTRACT

We performed a countrywide assessment of HIV drug resistance among 123 patients with virological failure on second-line antiretroviral therapy (ART) in Kenya. The percentage of patients harboring intermediate-to-high-level resistance was 27% for lopinavir-ritonavir, 24% for atazanavir-ritonavir and 7% for darunavir-ritonavir, and 25% had complete loss of activity to all available first- and second-line drugs. Overall, one in four patients failing second-line ART have completely exhausted available antiretrovirals in Kenya, highlighting the need for increased access to third-line drugs.

To date, nearly half a million HIV-1-infected patients in sub-Saharan Africa have been switched to second-line ART, based on boosted-protease-inhibitors (bPI), after first-line failure[1].With scale-up of viral-load testing, the number is forecasted to grow to 4-6 million by 2030, comprising 20% of all on ART[2]. Virological failure on second-line ART, mostly lopinavir-ritonavir-based, has been reported in up to 38% of patients after 3 years of treatment[3].However, data on resistance are limited and access to third-line ART is restricted due to exorbitantly high drug costs. In a cross-sectional study in the national ART program in Kenya, we assessed HIV drug resistance among patients failing second-line bPI-based ART between June 2010 and December 2015.

Treatment failure was defined as either clinico-immunological failure with a single confirmatory plasma viral load (pVL) of >1,000 cps/ml or two consecutive pVL >1,000 cps/ml after intensive adherence counseling. We included plasma/DBS specimens sent to the WHO-designated KEMRI/CDC laboratory for HIV drug resistance testing from ART sites in western Kenya (2010-2012) and nationwide (2013-2015). *Pol* gene sequences were obtained using the CDC in-house genotyping assay[4]. We calculated the genotypic susceptible scores (GSS) as 1.00-0.75-0.50-0.25 and 0, based on the Stanford HIV drug resistance algorithm v7.0: for susceptible, potential low-level-, low-level-, intermediate-level- and high-level resistance, respectively[5]. Predicted efficacy to WHO-recommended first-, second- and third-line regimens was calculated as an arithmetic sum of the individual-drug GSS; GSS of <2 was considered as exhaustion to the available drug options. Integrase-inhibitor (INSTI)-based regimens were assigned a full susceptibility score due to their limited use in the region. We compared the predicted GSS for potential third-line regimens based on the previous (INSTI+etravirine+darunavir-ritonavir)[6] and current (INSTI+darunavir-ritonavir+1 or 2 NRTIs)[7] WHO recommendations using the z-test. Factors associated with intermediate to high-level PI-resistance were assessed using multivariable logistic regression analyses. The study was approved by the scientific and ethics committees of the Kenya Medical Research Institute.

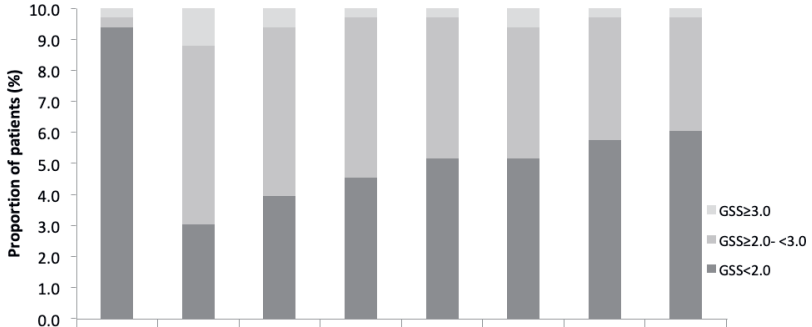
One hundred and twenty three out of 126 viral isolates had a successful genotype and were included in the analysis. The median age was 24 (IQR 10-36) years, median CD4 count was 114.5 (IQR 24-251) cells/μL, and mean VL was 4.8 (SD 0.1) log10 cps/mL. The median time on ART was 6.4 years (IQR 4.3-8.1), including 3.1 years (IQR 1.9-4.6) on second-line. One hundred and sixteen (97%)

patients were on lopinavir-ritonavir, with the most common NRTI-backbone being tenofovir+lamivudine (35%), followed by abacavir+lamivudine (23%), abacavir+didanosine (11%) and zidovudine+lamivudine (11%).

Sixty-three percent of patients had ≥1 NRTI resistance mutation, predominantly M184I/V (51%) and thymidine analogue mutations (TAMs) (37%). 32% of patients had ≥1 major PI resistance mutation with a median number of 3 (range 1-5), most frequently M46I/L (24%), I54V (22%) and V82A/T/F/S (20%). 24% of patients had triple-class (NNRTI, NRTI and PI) resistance, 34% had no NRTI or PI mutations, 18% had wild-type virus.

Twenty-seven percent of patients had intermediate-to-high level resistance to lopinavir-ritonavir, 24% to atazanavir-ritonavir and 7% to darunavir-ritonavir. Cross-resistance to the second-generation NNRTIs was present in 46% of patients for rilpivirine and 36% for etravirine. Of note, 25% (31/123) of the patients had exhausted all first- and second-line drug options available in Kenya (Figure 1).
Patients with PI-resistance were more likely to have ≥2TAMs (OR 15.1, 95%CI 5.3-42.9) but associations with duration of treatment, sex, age, CD4 and pVL were non-significant.

Predicted probability for having GSS more than 2 was highest if third-line regimens of darunavir-ritonavir along with INSTI, included etravirine as the third-agent (0.70). If etravirine was replaced with an NRTI-backbone the probabilities of GSS more than 2 were somewhat (although not statistically significantly) lower for dual NRTIs (zidovudine+lamivudine (0.61, p=0.219), tenofovir+lamivudine (0.55, p=0.102),and significantly lower for a single NRTI (lamivudine/emtricitabine (0.48, p=0.04), zidovudine (0.48, p=0.04), tenofovir (0.42, p=0.01 3), abacavir (0.39, p=0.007)) (Figure 1).



	Any 1st or 2nd line	ETR	3TC+AZT	TDF+3TC	3TC/FTC	AZT	TDF	ABC
GSS≥3.0	0.3	1.2	0.6	0.3	0.3	0.6	0.3	0.3
GSS≥2.0- <3.0	0.3	5.8	5.5	5.2	4.5	4.2	3.9	3.6
GSS<2.0	9.4	3.0	3.9	4.5	5.2	5.2	5.8	6.1

Figure 1: Predicted antiretroviral susceptibility to available WHO-recommended first-, second- and potential third-line regimens among patients failing second-line treatment in Kenya

Genotypic sensitivity scores (GSS) calculated as 1.00-0.75-0.50-0.25 and 0, based on Stanford HIV drug resistance algorithm categories for susceptible, potential low-level-, low-level-, intermediate-level- and high-level resistance respectively; GSS for combined ART calculated as arithmetic sum of individual drugs. 3TC-lamivudine; ABC-Abacavir; AZT-Zidovudine; ETR-etravirine; FTC-emtricitabine; TDF-tenofovir. Any 1st and 2nd -line includes NNRTIs NVP or EFV (1st-line), PIs lopinavir-ritonavir or atazanavir-ritonavir (2nd-line) with NRTI backbone of 3TC or FTC plus either AZT, ABC or TDF. The calculations for GSS in third-line include the core drugs INSTI+darunavir-ritonavir and the third agent as either etravirine (2nd generation NNRTI) or single or dual NRTI regimens as indicated in the x-axis.

This is among the first nationwide assessments of HIV drug resistance among patients failing second-line ART in sub-Saharan Africa. This study in the Kenyan national ART program suggests that about 27% of patients with second-line failure are in need of a switch to third-line therapy, with 25% demonstrating complete exhaustion of alternative first or second-line regimens. Few other observational studies in the African region have reported on ART exhaustion in 9-32% of patients failing second-line therapy[8–10]. These data indicate an urgent need for increasing access to third-line drugs, i.e. INSTIs (raltegravir, dolutegravir) and darunavir/ritonavir.

WHO-recommended third-line drugs are prohibitively expensive with costs nearly 6-14 times higher than the current first- and second-line regimens[11]. Sustainability is thus a challenge for ART programs in low and middle-income countries (LMICs), citing the case of Brazil where provision of third-line to about 5% of the patients accounts for ~40% of all ART resources[12]. Ongoing negotiations

with pharmaceutical companies for production of generic third-line options may potentially lead to price reductions in the near future[13].

About two-thirds of the participants did not have PI resistance mutations, which concurs with previous studies[10,14,15]. Possible explanations include: complete non-adherence hence no resistance mutations are selected in the absence of drugs; the characteristic short-mutant selection window for PIs, attributed to the rapid fall in the inhibitory concentration during non-adherence[16]; and mediation of PI-resistance by mutations outside the protease gene, specifically in the *gag*[17] and *env* genes[18]. In this study, we neither assessed the influence of these mutations nor that of adherence, hence we are unable to ascertain the cause of treatment failure in patients without major PI resistance mutations.

Due to limited data in support of NRTI-sparing regimens, WHO guidelines recommend recycling of NRTIs in third-line therapy. In our study, however, the predicted response for third-line regimens comprising INSTI plus darunavir/ritonavir was highest if it included etravirine as the third agent instead of a single NRTI, but was comparable with inclusion of 2 NRTIs in a four-drug combination. The low GSS of the NRTIs could be attributed to accumulation of TAMs, due to delayed switches. Optimal efficacy may thus depend on timely detection of failure and switch to third-line treatment.

Study limitation exists. We may have under-estimated the prevalence of second-line treatment failure as some ART sites may have been less vigilant, or lacked appropriate tools to timely identify these patients and confidently notify the national program. However, with the inclusion of routine viral-load tests and HIV drug resistance testing for second-line failures in recent guidelines[19,20], it is anticipated that patient identification will be significantly improved.

In conclusion, our study indicates that nearly one in four patients in Kenya failing second-line treatment has complete exhaustion to available antiretrovirals, emphasizing the need for increased access to third-line treatment in LMICs.

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Conflicts of interest

We declare that we have no conflicts of interest.

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Chapter 7

Primary resistance to integrase strand transfer inhibitors in patients infected with diverse HIV-1 subtypes in sub-Saharan Africa

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ABSTRACT

Objective

To investigate the prevalence and patterns of major and accessory resistance mutations associated with integrase strand transfer inhibitors (INSTI), across diverse HIV-1 subtypes in sub-Saharan Africa.

Methods

Pol gene sequences were obtained using Illumina next-generation sequencing (NGS) from 425 INSTI-naïve HIV-infected adults from Kenya (21.2%), Nigeria (7.3%), South Africa (22.8%), Uganda (25.2%) and Zambia (23.5%). Drug resistance interpretation was based on IAS 2017 mutation list and accessory mutations from Stanford HIVdb with resistance penalty scores of ≥10 to at least one INSTI. Resistance was further classified based on sensitivity thresholds of ≥20% (Sanger sequencing) and 1-20% for low-frequency variants (NGS).

Results

Of 425 genotypes, 48.7% were subtype C, 28.5% A, 10.1% D, 2.8% G, and 9.9% recombinants. Major INSTI resistance mutations were detected only at <20% threshold, at a prevalence of 2.4% (2.5% in subtype A, 2.4% C, 0% D, 8.3% G and 2.4% in recombinants) and included T66A/I (0.7%), E92G (0.5%), Y143C/S (0.7%), S147G (0.2%) and Q148R (0.5%). Accessory mutations occurred at a prevalence of 15.1% at the ≥20% threshold (23.1% in subtype A, 8.7% C, 11.6% D, 25% G and 23.8% in recombinants), and included L74I/M (10.4%), Q95K (0.5%), T97A (4%), E157Q (0.7%) and G163R/K (0.7%).

Conclusion

Major INSTI resistance mutations were rare and only occurred at low-level resistance detection thresholds. INSTI-based regimens are expected to be effective across the different major HIV-1 subtypes in the region.

INTRODUCTION

Since their first approval for clinical use in 2007, integrase strand transfer inhibitors (INSTI) for the treatment of HIV-1, have demonstrated a remarkable superiority over other drug classes in terms of safety and tolerability, fast viral clearance and high potency.[1] Three drugs have been currently approved: raltegravir, elvitegravir and dolutegravir with cabotegravir and bictegravir being in the final stages of clinical evaluation. Previously reserved as salvage therapy, they have since gained their way as the preferred first-line regimen as currently recommended by ART guidelines in high-income countries.[2,3] Their access in resource-limited settings has been restricted because of high prices, but recent collective bargains and agreements for development of generic dolutegravir, have seen their adoption into WHO guidelines as alternative first-line regimen.[4] To date, Botswana and Kenya are the only two countries in sub-Saharan Africa that are rolling out dolutegravir as part of standard-first-line ART. However, wide-scale access across the region is anticipated as generic drugs become available and as countries adopt the recent WHO guidelines recommending dolutegravir as the preferred first-line regimen in regions where the prevalence of pre-treatment NNRTI resistance is ≥10%.[5]

Despite high efficacy, resistance to INSTI is known to occur, leading to treatment failure.[6,7] To date >40 mutations have been associated with INSTI resistance, although many of them are thought to be polymorphisms.[8–10] The primary mutation pathways identified involve mainly substitutions at T66, E92 (elvitegravir), Y143 (elvitegravir plus raltegravir), Q148, N155 and R263 (elvitegravir, raltegravir and dolutegravir) amino acids.[8] In addition to these, some polymorphic mutations play a significant role in rescuing viral fitness and/or increasing the level of resistance.[8]

Biochemical studies have shown a subtype-dependent influence of natural polymorphisms on the occurrence and activity of INSTI resistance.[11–14] For instance, subtype B viruses have been shown to have a higher fitness cost associated with G118R, a recently proposed resistance pathway, compared to subtypes C and CRF_02 AG.[13] Consequently, G118R has rarely been observed in subtype B viruses. It has been postulated that G118R could be an alternative pathway for dolutegravir resistance in non-subtype B viruses, whereas R263K is the preferred pathway for subtype B viruses.[13] The occurrence of G118R is further

impacted by a rare natural polymorphism at codon 118 (GGG or GGA) that has a low genetic barrier, facilitating the transition of glycine to arginine (AGG or AGA) through a single point mutation.[15] The prevalence of the G118R mutation may also differ by subtype. Moreover, the impact of other polymorphic mutations in augmenting resistance and rescuing viral-fitness of the G118R mutants was also shown to be subtype dependent.[13] Taken together, these findings indicate that minor differences in polymorphisms between subtypes may influence the emergence of resistance variants, enhance resistance or even the development of new resistance pathways. Nonetheless, patient-derived pre-treatment data on INSTI resistance in sub-Saharan Africa remain scarce.

This study aimed to generate baseline data on INSTI resistance and polymorphisms across the various HIV-1 subtypes in sub-Saharan Africa, necessary for the rollout of INSTI-based regimens. To this end, we assessed the prevalence of natural occurring major and accessory resistance mutations, including those occurring at low frequencies, in a large international cohort of antiretroviral-naïve patients from five sub-Saharan African countries representing the major HIV-1 subtypes. Additionally, we also assessed the prevalence and subtype variation of the rare polymorphisms at the G118 position that may facilitate the occurrence of the G118R resistance pathway.

METHODS

Study design and population

The Pan-African Studies to Evaluate Resistance Monitoring (PASER-M) study was a prospective cohort of HIV-1-positive adults who initiated ART between 2007 and2009, at 13 clinical sites in 6 sub-Saharan African countries, as described previously.[16] We performed a case-control study (1:2 ratio) nested in the cohort to determine the clinical relevance of minority pre-treatment drug resistant variants. Cases were defined as patients who experienced virological failure after 12 months of first-line NNRTI-based ART (plasma viral-load of ≥400cps/mL). Controls were defined as patients who had viral suppression (plasma viral-load of <400 cps/ml) at month 12, matched by country, pre-treatment CD4 counts, viral-load and age.

The current study presents the analysis of 489 baseline samples collected from participants in the case-control study from 5 countries i.e. Kenya, Nigeria, South Africa, Uganda and Zambia.

Ethics

The study was approved by the national and local research ethics committees at the collaborating sites. Participants provided written informed consent at enrolment.

Virological analysis

Archived plasma samples were shipped to the IrsiCaixa laboratory (Badalona, Spain) for ultrasensitive genotyping using next-generation sequencing. Amplification was performed using the pan-HIV-1 *pol* assay described in Supplementary material. Briefly, viral RNA was extracted from plasma samples using the QiaAmp viral RNA mini kit following the manufacturer's instruction (Qiagen Inc, Chatsworth, CA, USA). Thereafter, a 3448 base pair segment of the 5' region of the pol gene was generated by a first RT-PCR one-step reaction using SuperScript™ III One-Step RT_PCR System with Platinum™ TaqDNA Polymerase (ThermoFisher Scientific Inc, Whaltham, MA, USA), followed by a nested PCR using Platinum™ Taq DNA Polymerase High Fidelity (ThermoFischer Scientific Inc, Whaltham, MA, USA). DNA libraries were then purified using Agencourt AMPure XP PCR Purification system (Beckman Coulter Inc, Brea, CA, USA), quantified using Quant-iT™ PicoGreen™ dsDNA Assay Kit (ThermoFisher Scientific Inc, Whaltham, MA, USA) and diluted. Diluted samples were then prepared for sequencing using the Nextera XT DNA Sample Preparation Kit and Nextera XT Index Kit (Illumina, San Diego, CA, USA), according to manufacturers' instructions. Finally, they were multiplexed into pools of 96 libraries and sequenced using the 500-cycle MiSeq Reagent Kits v.2 (Illumina, San Diego, CA, USA).

Assembly of the Miseq sequence reads (FASTQ) and quality assessment was done using PASeq (https://paseq.org), an automated HIV drug resistance analysis pipeline (IrsiCaixa, Barcelona, Spain). We then generated a list of amino acid substitutions relative to the reference sequence (HXB2 for non-C subtypes, and 97ZA012 for subtype C viruses). INSTI resistance mutations were defined as 1) major resistance mutations according to the IAS 2017 mutation list,[17] and 2) accessory mutations as identified by the IAS 2017 mutation list and with a Stanford HIVdb ≥10 resistance penalty score.[18] Major resistance mutations are those that cause significant reduction in INSTI susceptibility even when they

occur alone. Accessory mutations are those that cause only low-level reduction of INSTI susceptibility when they occur alone, but may serve to augment resistance or/and restore fitness of viral mutants having major resistance mutations.

Resistance was reported at sensitivity thresholds of i) ≥20% as used with standard Sanger sequencing and ii) the cumulative resistance that includes mutations at sensitivity thresholds of 1%, 2%, 5% and 10%, as there is limited understanding on the role of minority INSTI resistance variants.

Additionally, we assessed subtype variability for the rare polymorphisms (GGG and GGA), which influences the occurrence of the G118R mutation resistance pathway. HIV subtyping was done by the REGA v3.0 subtyping tool.[19] All sequences in this study have been deposited in GenBank (accession nos. MG693785-MG694209).

Differences in DRM distributions by subtype were assessed by Chi-square statistic and fisher-exact test.

RESULTS

Patient characteristics

Of 489 samples, 425 (87%) were successfully sequenced and analyzed; 28 (6%) failed amplification and 36 (7%) either failed sequencing or had a poor-quality sequence. The geographic distribution of successful genotypes was: Uganda (107, 25.2%), Zambia (100, 23.5%), South Africa (97, 22.8%), Kenya (90, 21.2%) and Nigeria (31, 7.3%). Representation of each country was close to that of the original PASER-M cohort; Kenya (424, 16.4%), Nigeria (193, 7.5%), South Africa (601, 23.3%), Uganda (606, 23.5%) and Zambia (551, 21.4%). 59.5% (253) were women, median age was 36 years (IQR 30-42), and median CD4 count was 119.0 (IQR 53.0-192.5) cells/mm^3. The distribution of HIV-1 subtypes was C (207, 48.7%), followed by A (121, 28.5%), D (43, 10.1%), recombinants forms (42, 9.9%) and G (12, 2.8%).

Prevalence of major INSTI resistance

We did not observe any major INSTI mutation at the resistance detection threshold of ≥20%. The cumulative prevalence of major INSTI mutations including those circulating at low-level frequency was 0.2% (1), 0.5% (2), 0.7% (3) and 2.4% (10) at sensitivity thresholds of 10%, 5%, 2% and 1% respectively (Figure 1). The pattern of major resistance mutations at <20% threshold was: T66A/I (3, 0.7%), E92G (2, 0.5%), Y143C/S (3, 0.7%), S147G (1, 0.2%) and Q148R (2, 0.5%).

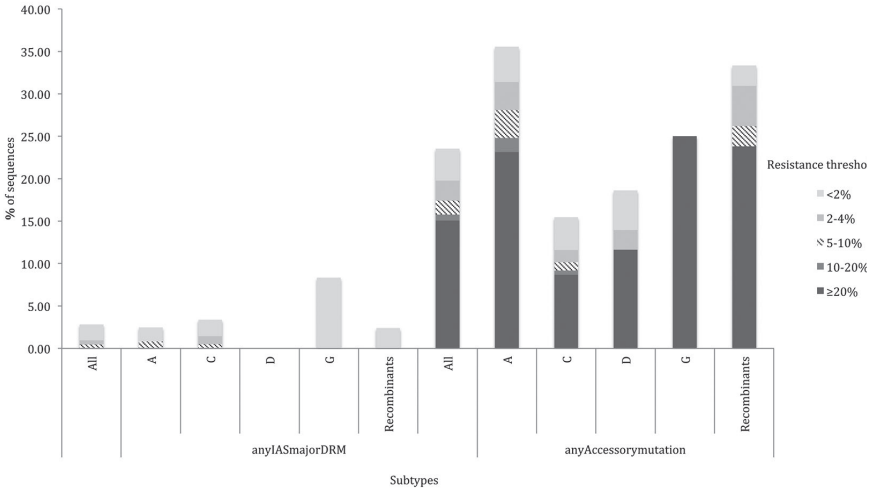

Figure 1: Patterns of primary INSTI resistance mutations across major subtypes in sub-Saharan Africa. All primary resistance mutations occurred as minority variants predominantly at frequencies of <2%

Patterns of accessory INSTI mutations

The prevalence of accessory INSTI mutations was 15.1% (64) at the resistance detection threshold of ≥20%. The cumulative prevalence of accessory mutations including those at low-level frequency was 15.8% (67), 17.4% (74), 20.0% (85) and 23.3% (99) at sensitivity thresholds of 10%, 5%, 2% and 1% respectively (Figure 1). L74I/M and T97A were the most common accessory INSTI mutations and occurred at a prevalence of 10.4% (44) and 4.0% (17) respectively at the ≥20% detection threshold and 13.4% (57) and 6.8% (29) at the ≥1% threshold, respectively (Table 1). Other mutations occurring at ≥20% detection threshold were Q95K (2, 0.5%), E157Q (3, 0.7%) and G163R/K (3, 0.7%).

Sequences from 3.5% (15) participants harbored > 1 drug resistance mutation, which comprised mainly of L74I/M and T97A accessory mutations (Table S1).

Patterns of INSTI resistance by subtype

We observed a variation in the patterns of both major and accessory mutations by subtype (Table 1). Cumulative prevalence at the 1% threshold for any major mutations was 8.3% (1) in subtype G, 2.4% (5) in C, 2.5 % (3) in A, 2.4% (1) for recombinants and none in D. The differences however were not of statistical significance (p=0.450). The major INSTI mutations, E92G and Y143C/S were

only observed in subtype A; Q148R in subtype C; and S147G in subtype G. The cumulative prevalence of accessory mutations at ≥20% detection threshold was highest in subtype G (25%, 3), followed by recombinants (23.8%, 10), A (23.1%, 28), D (11.6%, 5) and subtype C (8.7%, 18). Compared with subtype C, accessory mutations detected at ≥20% threshold, occurred at statistically significant higher proportions in subtypes A ($p<0.001$) and in recombinants ($p=0.009$). The cumulative prevalence of accessory mutations including those at low-level frequency (≥1%) was highest in subtype A (35.5%, 43), followed by recombinants (33.3%, 14), G (25%, 3), D (18.6%, 14) and 15% (31) for subtype C. L74I/M occurred mainly among participants with subtype A (14.0% and 18.2%), subtype G (16.7% and 16.7%) and recombinant forms (14.3% and 16.7%) at the ≥20% and ≥1% detection thresholds respectively. T97A equally occurred at the highest prevalence among participants infected with HIV-1 subtype A, at both the 20% and 1% detection thresholds, i.e. 9.1% and 16.5% respectively.

Table 1: Comparative analysis of INSTI major and accessory mutations across the major HIV-1 subtypes in sub-Saharan Africa

			Proportion (%)of patients with detected mutation[a]											
			All subtypes (n=425)		Subtype A (n=121)		Subtype C (n=207)		Subtype D (n=43)		Subtype G (n=12)		Recombinants[b] (n=42)	
AA	Pos	Mut	≥20	≥1	≥20	≥1	≥20	≥1	≥20	≥1	≥20	≥1	≥20	≥1
Any major mut			-	2.4	-	2.5	-	2.4	-	-	-	8.3	-	2.4
Any accessory mut			15.1	23.3	23.1	35.5	8.7	15.0	11.6	18.6	25.0	25.0	23.8	33.3
T	**66**	**I/A**	-	0.7	-	0.8	-	0.5	-	-	-	-	-	2.4
L	74	I/M	10.4	13.4	14.0	18.2	7.7	10.1	7.0	11.6	16.7	16.7	14.3	16.7
E	**92**	G	-	0.5	-	1.7	-	-	-	-	-	-	-	-
Q	95	K	0.5	0.7	-	-	-	0.5	-	-	-	-	4.8	4.8
T	97	A	4.0	6.8	9.1	16.5	1.0	1.9	2.3	2.3	8.3	8.3	4.8	7.1
G	118	R	-	0.2	-	-	-	0.5						
E	138	A/K/T	-	1.2	-	4.1	-	-	-	-	-	-	-	-
Y	**143**	**C/S**	-	0.7	-	0.8	-	-	-	-	-	-	-	-
P	145	S	-	0.2	-	-	-	-	-	-	-	-	-	2.4
S	**147**	**G**	-	0.2	-	-	-	-	-	-	-	8.3	-	-
Q	**148**	**R**	-	0.5	-	-	-	1.0	-	-	-	-	-	-
V	151	A	-	0.5	-	0.8	-	0.5	-	-	-	-	-	-
S	153	Y	-	0.2	-	-	-	0.5	-	-	-	-	-	-
E	157	Q	0.7	0.9	-	-	0.5	1.0	2.3	2.3	-	-	2.4	2.4
G	163	R/K	0.7	1.6	1.7	1.7	-	0.5	2.3	4.7	-	-	-	4.8
S	230	R	-	0.5	-	-	-	0.5	-	-	-	-	-	2.4
R	263	K	-	0.2	-	-	-	0.5	-	-	-	-	-	-

WT wild-type; Pos position; Mut mutation; **bold** indicates primary INSTI mutations, - indicates not [a]Resistance thresholds at 20 and 1%, detected [b]Recombinants AD (n=14) CRF_02AG (n=9), AC (n=6) AG (n=4), AG complex recombinants (n=6) DG (n=1), CD (n=2),

Natural polymorphisms at codon G118

The overall frequency of polymorphisms at G118 was 6.1% (26) being 4% (17) for GGA and 2.1% (9) for GGG (Table 2). The frequency of the GGA motif was highest among subtype A genotypes (6.6%, 8) while the GGG was highest among the recombinant viruses (9.5%, 4). Subtype D had the lowest frequency for the rare polymorphisms, having only 1 participant with the GGA motif (2.3%), while none was present in subtype G.

Table 2: Analysis of codon usage at position 118 across the major HIV-1 subtypes in sub-Saharan Africa

	Glycine codon % (n)			
Subtype (n)	[a]GGA	[a]GGG	GGC	GGT
All (425)	4 (17)	2.1 (9)	83.1 (353)	10.8 (46)
A (121)	6.6 (8)	1.7 (2)	85.1 (103)	6.6 (8)
C (207)	3.4 (7)	1.4 (3)	79.2 (164)	15.9 (33)
D (43)	2.3 (1)	-	97.7 (42)	-
G	-	-	91.7 (11)	8.3 (1)
[b]Recombinants (42)	2.4 (1)	9.5 (4)	78.6 (33)	9.5 (4)

[a] Rare low-genetic barrier polymorphisms;
[b] Recombinants AD (14) CRF_02AG (9), AC (6) AG (4), AG complex recombinants (6) DG (1), CD (2).

DISCUSSION

This observational study among patients enrolled in a large multi-country African cohort showed a low prevalence of major INSTI resistance mutations, present only at a low-level detection threshold of <20%. This is one of the first large-scale studies in Africa to assess background resistance to INSTI drugs. Although the samples analyzed date back to 2007-2009, the findings are still highly relevant for today, given that the use of INSTIs in the region has been virtually non-existent over the past decade. Our findings provide important evidence to guide the wide-scale implementation of dolutegravir-based regimen in the region. Recent data from WHO surveys assessing pre-treatment drug resistance (PDR) found that in 3 out of 4 countries surveyed, >10% of people who were newly initiating ART had HIV strains resistant to NNRTIs. Subsequently, WHO issued new guidelines recommending a rapid transition to dolutegravir-based first-line treatment in countries with a pretreatment drug resistance prevalence of ≥10% and in persons with previous ARV exposure.[5,20] Moreover more countries are expected to transition to dolutegravir-based treatment, regardless of the prevalence of pre-treatment

NNRTI resistance, due to availability of low-cost generic fixed-dose combination; (tenofovir/lamivudine/dolutegravir, TLD, USD 75 per person per year). [20]

Similar findings for the occurrence of low-frequency major resistance mutations in INSTI-naïve patients have previously been reported, but the clinical relevance remains uncertain.[21,22] A secondary analysis of patients participating in the BENCHMRK-1 raltegravir clinical trial in North and South America, found a higher but non-significant prevalence of baseline INSTI major mutations (Y143R/C/H, Q148R/H/K and N155H) in patients with virological failure compared to those with sustained virological suppression, which was present only at frequencies of <1%.[21] Another study on INSTI-naïve patients initiating raltegravir-based ART also found a high prevalence of Q148R variants which were present mainly at frequency levels of <1%,[22] with no significant impact on virological response.[22] However, both studies were limited by a small sample size and restricted to HIV-1 subtype B.

Accessory resistance mutations occurred mainly at a higher resistance detection threshold (≥20%) and included L74I/M, Q95K, T97A, E157Q and G163R/K. L74I/M and T97A were the most common accessory mutations and similar to previous reports, were more frequent in participants infected with subtype A, G and recombinant viruses.[23,24] L74I/M are polymorphic mutations commonly selected by all the three INSTI drugs and also occurs in levels of between 0.5 and20% in untreated populations with high prevalence in subtype A, G and A/G recombinants.[23] It however does not affect INSTI susceptibility unless in combination with other major INSTI resistance mutations, mainly the Q148H/K/R mutation.[18] T97A on the other hand is mainly selected among patients on raltegravir and elvitegravir and occurs in 1-5% of untreated patients.[23] Similarly, T97A has minimal effect on INSTI susceptibility on its own but leads to significant reduction in raltegravir and elvitegravir susceptibility in combination with Y143 and N155H major resistance mutations.[18] Q95K is a non-polymorphic accessory mutation selected by patients on raltegravir and elvitegravir.[23] On its own it does not affect INSTI susceptibility but enhances resistance of N155H mutants to raltegravir and elvitegravir.[18,25] E157Q is a polymorphic accessory mutation selected in-vitro by elvitegravir and in raltegravir treated patients.[23] On its own, E157Q has no significant effect on INSTI susceptibility but reduces susceptibility of raltegravir and elvitegravir in the presence of N155H major resistance mutation.[18] A recent in-vitro study showed that it could also play a compensatory role in restoring integrase activity of R263K mutants as well as

augmenting their resistance to dolutegravir.[26] G163R/K on the other hand is a non-polymorphic accessory mutation occurring naturally mainly in subtype F viruses but is commonly selected in raltegravir-treated patients.[23] On its own it has minimal effect on INSTI susceptibility but increases resistance to raltegravir and elvitegravir when occurring jointly with Y143 and N155H mutations.[18]

There is a need to monitor continuously for new resistance patterns that may have not been previously observed among subtype B viruses. Recent studies have suggested an alternative resistance pathway for dolutegravir that involves the G118R mutation selected mainly in non-subtype B viruses.[13] An in-depth analysis revealed that this could be facilitated by rare polymorphisms at the G118 position, which requires a single-point transition mutation from glycine (GGA or GGG) to arginine (AGA or AGG).[15] This is in contrast to the dominant polymorphisms GGC and GGT that require either a two point mutation (GGC→AGA or GGT→AGA) or a single-step unfavorable transversion (GGC→CGC and GGT→CGT, respectively). Moreover, the rare polymorphism at the G118 position is also an APOBEC3G/F hotspot, and may thus be amenable to APOBEC3G/F mediated G to A transitions leading to resistance.[27] A previous analysis from Los Alamos database showed that the rare G118 polymorphisms occur at a low prevalence of 2.5% ranging from 0 for subtypes F and G to 1.4% for subtype B, to 3.8% in CRF01_AE recombinants.[15] In our study, however, the overall frequency was at 6.1% with frequencies as high as 8.3% and 11.9% in subtypes A and recombinants respectively. These findings may suggest a higher propensity for occurrence of this mutation in non-subtype B viruses. When the G118R occurs together with the T66I/A and E138K mutations, this results in a 6.5-fold resistance to dolutegravir.[15] These findings indicate the need for close monitoring of dolutegravir implementation in the region, as there is still inadequate information on INSTI resistance mutations that may lead to treatment failure in patients infected with non-subtype B viruses.

There are some study limitations. The nested case-control design may not provide a true random sample of the total cohort and may have led to under-representation of the major HIV-1 subtypes. This limits our ability to generalize our results, which is however partially compensated by the large sample size.

Altogether these findings provide some reassurance on the potential effectiveness of INSTI-based regimens in these settings, as more countries adapt and implement the recent guidelines advocating for dolutegravir based first-

line treatment. However, real-life data on treatment response and resistance mutations selected by dolutegravir in non-B subtypes are still required. This is particularly important citing the possibility of new subtype dependent resistance pathways like the G118R, which may considerably impact on the efficacy of dolutegravir. Further studies are needed to understand the potential impact of the low-frequency INSTI resistance mutations observed in this and other studies. In addition, continuous surveillance for both transmitted and acquired resistance is also necessary following reports from Western countries showing the emergence of INSTI resistance in both naïve and treated patients.[6,7,28,29]

In conclusion, we observed a low prevalence of major INSTI resistance mutations, which occurred only at low-level detection threshold. These findings offer some reassurance for the effectiveness of INSTI-based regimens in sub-Saharan Africa across the different major circulating HIV-1 subtypes.

Transparency declarations

All authors declare that they have no conflict of interest.

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Authors' contributions

TFRW is the PASER principal investigator. RP, TFRW and RLH conceived the study. SCI, MN-J, MC and MP perfomed the laboratory testing and data analysis. SCI, MP, TFRW and RLH drafted the manuscript. All authors provided valuable input to interpretation of the data and critically reviewed the paper for important intellectual content. All authors reviewed and approved the final version of the manuscript.

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Supplementary Table 1: Resistance profile of patients with multiple INSTI drug resistance mutations

	Major INSTI [a]DRM (%, frequency of detection)	Accessory INSTI [a]DRM (%, frequency of detection)	Subtype
001	-	T97A (69.8), P145S (1.1)	D/G
002		T97A (85.5), E138K (1.2)	A
003		T97A (10.4), L74M (21.3)	A
004		T97A (99.9), L74I (100)	cpx
005	Y143C (1.3)	T97A (28.3)	C
006		T97A (1.9), L74I (88.1), L74M (2.5)	C
007		T97A (83.9), G163R (51.8)	A
008		T97A (10.4), E138K (2.5)	A
009		T97A (84.1), L74I (52.7)	A
010	Y143S (1.6), E92G (1.0)	T97A (58.3)	A
011		T97A (99.4), L74I (99.3)	D
012		T97A (3.7), L74I (99.8)	A/D
013		T97A (22.9), L74I (12.0)	A
014		L74I (38.3), L74M (35.4), G163R (1.2)	C
015		L74I (99.8), E138K (1.7)	A

[a] DRM; drug resistant mutations

Chapter 8

Curbing the impact and rise of HIV drug resistance in low- and middle-income countries: the role of dolutegravir-containing regimens

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ABSTRACT

To improve virological suppression and address the emerging threat of HIV drug resistance (HIVDR), many low-and middle-income countries (LMIC) are moving away from non-nucleoside reverse transcriptase inhibitors (NNRTI) and transitioning to dolutegravir (DTG) as part of a more affordable standard first- and second-line antiretroviral therapy (ART). Whereas this transition may blunt the impact of rising NNRTI resistance and yield improved ART outcomes, the transition presents new challenges. First, current safety concerns for DTG use in women of reproductive age may imply the need for an alternative solution to HIVDR. Second, pre-existing resistance to the co-administered NRTI backbone may reduce effectiveness and durability of DTG, potentially further augmented when access to viral load tests is limited. Third, there is limited information on the genetic correlates of resistance to DTG, particularly in patients infected with HIV-1 non-B subtypes. Finally, clinical management of patients who experience virological failure on a DTG-based regimen will pose challenges due to uncertainty of whether DTG resistance has actually developed and switching is needed or whether improved adherence is sufficient. These considerations should be addressed to consolidate expected gains from widespread introduction of DTG in LMIC.

INTRODUCTION

The past decade has witnessed an unprecedented scale-up of access to life-saving antiretroviral treatment (ART) for persons infected with HIV-1, which has dramatically reduced infectiousness and improved health and life expectancy of millions of people.[1] The widely adopted public health approach to ART, recommended by the World Health Organization (WHO), has been largely based on using a dual nucleoside RT inhibitor (NRTI) backbone in combination with a companion non-nucleoside reverse transcriptase inhibitor (NNRTI), either efavirenz and/or nevirapine. However, evidence is emerging globally that HIV variants resistant to the NNRTI class are on the rise in populations initiating ART, so-called pretreatment drug resistance (PDR).[2–5] Recent surveys from WHO suggest that in several low-and middle-income countries (LIMC) over 1 in 10 HIV-infected patients initiating ART have PDR to efavirenz and/or nevirapine.[5] PDR is associated with poor virological outcomes, impaired immune recovery, reduced durability of current NNRTI-based first-line regimens, and increased mortality in both adults and children.[6–10] The rise in PDR has been forecasted to drive an increase in mortality, HIV incidence and overall ART programmatic costs, if changes to HIV treatment regimens are not made.[11]

Since 2014, dolutegravir (DTG), a second-generation strand transfer inhibitor (INSTI), has been increasingly used as part of first-line regimens in high-income settings[12,13] because of its favorable efficacy and safety profile. In LMIC, where until recently its use remained restricted due to high costs, a new low-cost generic fixed-dose combination of tenofovir disoproxil fumarate, lamivudine, and DTG 300/300/50 mg ("TLD") has been made available at an affordable price. Since September 2017, 92 LMICs are licensed through the Medicines Patent Pool to obtain TLD at a median price of US $75 per person per year. [14] This is comparable or even lower than NNRTI-based ART[14]. WHO has recognized TLD use as an attractive opportunity to further improve virological suppression rates and address or mitigate concerns raised by increasing levels of drug resistance to NNRTI-based first-line ART.[15] As a result, WHO has recently issued interim guidelines recommending the use of DTG in first and second-line treatment as well as in patients currently on alternative treatment.[15] The US President's Emergency Plan For AIDS Relief (PEPFAR) also initiated accelerated access of TLD in all HIV-infected patients [16] in PEPFAR-supported countries to maximize its benefits and minimize programmatic logistics for provision of multiple drugs.

Despite these optimistic perspectives, there are several notes of caution to heed if DTG would be positioned as the single overall 'solution' to the rise in HIVDR in LMIC. In this Viewpoint, we discuss the potential public health opportunities and challenges of the expanded use of DTG-based ART in LMIC with an emphasis on HIVDR. We review available data and knowledge gaps on its resistance profile, in the context of the public health approach to ART and HIV-1 non-B subtype infections, and highlight the continuous need for a solid HIVDR surveillance and prevention framework and stringent therapeutic monitoring strategies.

Dolutegravir efficacy, safety and tolerability

DTG is a second-generation integrase strand transfer inhibitor (INSTI) drug with a superior efficacy to other first-line drugs including efavirenz, atazanavir, and darunavir, and is non-inferior to the first generation INSTI raltegravir.[17–21] In addition, DTG is reportedly a more potent second-line therapy compared to ritonavir-boosted lopinavir when used with at least one fully-active NRTI drug[22]. A twice-daily dosage was demonstrated effective in INSTI experienced patients with minimal resistance to DTG[23,24], indicating its potential for use in salvage therapy. Recently, DTG dual therapy with lamivudine was shown to be a promising strategy when used as maintenance therapy in patients with viral suppression [25]. In addition DTG was non-inferior to triple-drug ART when used in combination with lamivudine as dual-therapy first-line regimen, in antiretroviral-naive patients with a plasma viral load of ≤500,000 cps/ml.[26] Initial speculations that DTG could be used as mono-therapy were refuted by studies that indicated an increased risk of virological failure, combined with INSTI-resistance.[27,28] In addition to a high efficacy, DTG has a good tolerability, [29] and a higher genetic barrier to resistance. Moreover, DTG has become increasingly affordable in fixed dose combinations (FDC).[14]

Safety concerns limits DTG use in specific populations

A recent report from Botswana highlighted potential safety concerns related to an increased risk of neural tube defects in infants born to women who were taking DTG at the time of conception.[30] Following this, the recent WHO interim guidelines recommend the use of DTG in women of reproductive age only when a consistent and reliable contraception is assured.[15] Adopting this recommendation may imply the need for an alternative regimen for most of the patients, especially those in sub-Saharan Africa, where the population of women of reproductive age comprise 60-70% of people living with HIV, and access to effective contraception is limited.[31]

The 2017 WHO report showed that in LMIC, PDR particularly associated with NNRTI in women is nearly two times higher than in men and had exceeded 10% in 8 of the 11 countries surveyed.[32] This suggests that alternative solutions may be needed for women, pending further confirmation of observed safety concerns. In the meanwhile, it remains prudent for countries to assess population levels of PDR to select the most effective regimen for use, particularly in women.

In addition to the above, a recent meta-analysis of data from four clinical trials showed significantly high rates of adverse events and treatment discontinuation in patients switched from other regimens to DTG.[33] Overall these findings highlight the need for enhanced pharmacovigilance and the provision of alternative ART, when rolling out DTG in LMICs.

Limited information on DTG resistance mutation patterns

To date, most patients who accessed DTG are from high-income settings infected with HIV-1 subtype B. In these settings, among treatment-naïve patients only two cases of possible DTG resistance have been reported to date. The first case was a late presenter with high viremia who started on tenofovir, emtricitabine, DTG and experienced viral rebound within two weeks of treatment with a transient Met184Ile RT mutation detected at ~3 weeks, and a Gln148Lys INSTI mutation at 5 weeks.[34] Of note, baseline INSTI resistance testing was not done, and it is possible that the Gln148Lys was already transmitted during infection. Subsequently, the patient maintained viral suppression with an optimized background regimen rilpivirine, tenofovir and emtricitabine. The second case was a patient enrolled in the ACTG5353 study assessing the efficacy of DTG plus 3TC dual combination in treatment-naïve individuals.[35] The patient achieved viral suppression by 4 weeks of treatment, but experienced virological failure by week 8 with Met184Val RT and Arg263Lys INSTI mutations being detected at 16 weeks.

In treatment-experienced but INSTI-naïve patients, a few additional cases have been reported to experience virological failure with DTG resistance mutations.[36,37] In particular, the Arg263Lys INSTI mutation has been reported in 4 patients (2 in subtype B and 2 in C) , Asn155His INSTI mutation in two patients infected with a non-B subtype virus, Gly118Arg in two patients (1 subtype B and 1 C), and Glu138Glu/Lys and His51His/Tyr in one patient infected with subtype C virus.

Patients failing on DTG mono-therapy were shown to further select for the Gln148His/Arg/Lys INSTI mutations accompanied by compensatory mutations leading to intermediate- to high-level DTG resistance.[38]

Among INSTI-experienced patients, the Gln148 mutation together with 2 or more accessory mutations significantly impairs DTG efficacy,[23,39,40] although the use of a twice-daily DTG dosage can significantly improve treatment response in patients with fewer mutations.[23,24]

There is limited information on the patterns of DTG resistance in non-B subtypes, although available data suggest the likelihood of HIV-1 subtype influencing the mutational patterns of INSTI resistance.[37,41–44] *In vitro* studies have shown that the Arg263Lys INSTI mutation is mainly selected in viral isolates from subtype B and Gly118Arg in non-B subtypes.[42,45] Selection of Gly118Arg is possibly influenced by the presence of a rare polymorphism with a low genetic barrier[45] which could be particularly common in patients infected with subtype A.[46] This could result in differential sub-type specific DTG-associated resistance prevalence *in vivo*, as has been observed with high prevalence of the Lys65Arg mutation associated with tenofovir resistance in subtype C.[47,48]

DTG replacement risks among patients with NRTI resistance

Resistance to the NRTI backbone is very common among patients with virological failure on NNRTI-based first-line ART in LMIC: in the TenoRes study involving cohorts from 36 countries globally, 57% have documented tenofovir resistance and of those with tenofovir resistance, 83% have also resistance to emtricitabine and lamivudine.[48] Therefore, if patients are switched from a first- or second-line regimen to a DTG-based regimen in the absence of virological monitoring, there is a risk that those patients failing with dual backbone resistance could be using a functional DTG monotherapy. Recent studies evaluating DTG mono-therapy in maintenance strategies have reported INSTI resistance in 50-82% of patients with virological failure.[38,49] Therefore, these data support an approach of DTG replacements be performed only when virological suppression is confirmed.

Recent data from the DAWNING study comparing DTG with ritonavir-boosted LPV in patients failing first-line NNRTI-based ART demonstrated high efficacy of 84% among patients with less than 2 fully active NRTIs. This suggests adequate residual NRTI activity when at least one NRTI remained unaffected by resistance mutations. [50] Such findings are similar to what has been reported with bPI-based regimens.[51] Further analysis however showed a reduced efficacy of 76% for patients maintained on NRTI drugs used in first-line ART compared to 87% for those who

were switched to newer NRTIs according to the WHO recommendation.[52] This suggests the need for optimization of the NRTI backbone when DTG is used in second-line as also recommended in the recent WHO guidelines.[15] More studies are needed to inform optimal strategies for recycling NRTIs with DTG-based ART.

To mitigate the risk of DTG resistance, PEPFAR recommends that ART programs should closely monitor treatment response by use of a viral-load test within 3-6 months of switching. [16,53] However, in LMIC this poses a substantial challenge, since many ART programs currently do not provide universal access to routine virological monitoring. As of July 2018, only 50% of the patients on ART in LMIC were estimated to have received at least one VL test in the past year.[54] In another report on 7 African countries, substantial differences were observed with respect to access to VL testing for ART patients: from 91% in Namibia, to 5% in Tanzania.[55] In settings where switch to DTG will be done in absence of viral load testing, WHO recommends close monitoring of treatment outcomes including viral load and drug resistance by use of well-designed cohorts or national representative surveys.[15]

Moreover, even in those settings where VL testing would be routinely used, the WHO-recommended cut-off of a plasma viral load of 1,000 copies/ml to trigger a regimen switch could result in late detection of failure with the risk of accumulation of HIVDR.[38,43,56,57]

Additionally, programmatic challenges documented in LMIC (e.g. drug stock out, poor retention, poor adherence, etc.) associated with suboptimal therapeutic response will not disappear in the presence of DTG. Therefore, higher rates of VF and the potential for HIVDR could be expected in LMIC compared to that observed in controlled trial settings.

Finally, in the absence of individualized resistance testing to optimize selection of the NRTI backbone, it remains unclear how a WHO recommended optimized NRTI backbone may impact on the durability of DTG-based therapy. Further research is clearly needed to monitor the durability of DTG-based ART and resistance patterns across the different proposed groups in LMIC and the impact of NRTI resistance on a DTG-containing regimen.

Change to DTG warrants optimal switching strategy

The WHO switching algorithm currently recommends the use of a confirmed VL>1,000 c/ml to trigger a change in regimen and move from NNRTI to the more costly boosted PI-based second line ART.[57] Approximately 20% of people on NNRTI-based ART have a VL>1,000 c/ml 12 months after ART initiation.[5,58] Among those failures, between 70 to 90% harbor high-level resistant variants[5,48], warranting a need for timely switch to second- line ART if they remain unsuppressed after enhanced adherence intervention. On the contrary data from clinical trials indicate that of the 10-18% of patients who initiated first-line DTG-based ART experiencing virological failure within 48 weeks of treatment,[17–22] most do not harbor any resistance to either the INSTI or NRTI backbone.[17–22]

This difference in resistance prevalence prompts considerations on the appropriateness of applying the current switching guidelines for managing VF on a NNRTI-containing regimen to patients experiencing VF on a TLD regimen. Therefore, studies will be needed to determine the frequency and mechanisms of VF on a first-line TLD regimen in routine LMIC settings. Knowing the frequency with which patients experience VF during first-line TLD, have resistance to the cytosine analogues, TDF, and DTG components of the regimen, re-suppress after intensive adherence counseling, will be required to optimize the management of VF in regions where routine genotypic resistance testing is not available. Preliminary studies have suggested possible alternative mechanisms for DTG resistance outside the integrase gene [59] [60] but further research is needed. Where possible, individualized resistance tests could help to optimize the composition of the NRTI background and help preventing premature and unnecessary switches to more costly PI-based regimens. It is worth noting that countries like Botswana and Brazil have policies recommending the use of individual resistance testing to guide the clinical management of patients with virological failure on DTG-based regimens.[61]

Rational antiretroviral drug sequencing

An additional concern relates to optimal drug sequencing strategies in patients experiencing treatment failure with DTG-based treatment, given the limited drug options in many LMIC. The current WHO-recommended sequencing approach of ART regimens in adults and adolescents is a standard first-line regimen of a preferred NNRTI (efavirenz) with a dual NRTI-backbone, followed by a second-line regimen of a ritonavir-boosted PI (either ATV or LPV) with 1 or 2 unused or recycled NRTIs, and followed by third-line of an INSTI combined with ritonavir-boosted darunavir

with or without 1 or 2 optimized NRTIs. In the 2018 interim WHO guidelines, the alternative sequencing approach is a DTG-based first-line regimen, followed by a bPI-based second-line, and ritonavir-boosted darunavir in third-line, with recycled DTG and 1 or 2 NRTIs, preferably optimized based on resistance test. [15] While DTG is likely to have residual activity when recycled with fully active DRV-r in third-line, further research is still warranted to assess the efficacy of this approach.

On the other hand, it is generally expected that the use of DTG in first-line would lead to fewer cases of treatment failure and reduce the need for next-lines of treatment. In short, the use of DTG-based first-line regimens may reduce the available sequential treatment lines but may increase the durability of first line and brings focus to the potential need for individualized resistance testing as part of treatment monitoring.

The continued necessity for population-based resistance surveillance in LMICs

Independent of the timeliness and success of introduction of DTG in LMICs, continued resistance surveillance remains indicated (**Table**). First, HIV drug resistance is one of the markers of success or failure of HIV programs. There is a continuous need to monitor critical gaps within HIV programs that facilitate the occurrence of resistance. These include quality indicators such as viral load suppression rates, drug stock outs, patient uptake and retention, pharmacy pick-up rates, as included in the WHO-defined early warning indicators for HIV drug resistance.[57] Monitoring these quality indicators, as part of programme monitoring and evaluation efforts, will continue to provide important information at the programmatic and clinic level ART that help identifying gaps to be addressed to curb wide-scale emergence of resistance.

The implementation of laboratory-based nationally representative surveys of population-level HIV drug resistance both in untreated and treated populations will be important to provide up-to-date information to guide and if needed accelerate transitions from NNRTI-based first-line. Moreover, such surveys will be able to monitor any future emergence of transmitted DTG resistance. These surveys will continue documenting NRTI resistance, especially in patients failing NRTI-based pre-exposure prophylaxis. Finally, such surveys can also inform on optimum individual management of patients failing DTG-based treatment including the use of resistance tests.

Table: Resistance assessment needs in the era of dolutegravir-based antiretroviral therapy in low- and middle-income countries

	Rationale	Strategy		Public-health action
		Population-based DRT	Individual-level DRT	
NRTI resistance	Possible impact on dolutegravir efficacy	a. PDR survey in patients initiating or re-initiating ART b. PDR surveillance in patients failing on tenofovir -based PrEP c. HIVDR surveillance in children <18 months	Patients with virological failure switching to dolutegravir-based treatment	-Perform viral load testing before switching to dolutegravir based regimen -If virological failure is detected, optimize use of the most effective NRTI backbone
Dolutegravir resistance	Possible increased frequency of dolutegravir resistance in non-B subtypes	a. Pretreatment drug resistance to detect transmitted resistance to integrase-inhibitor based regimens b. Acquired drug resistance to assess prevalence and patterns of dolutegravir resistance in failing patients	Patients with virological failure, to prevent unnecessary switches and optimize next-line of treatment	Determine and plan for alternative regimens and effective drug sequencing strategies
NNRTI resistance	Potential for NNRTI use in specific populations i.e. a) countries with limited access to low-cost dolutegravir based regimen b)Women of child-bearing potential in LMICs when effective and reliable contraceptives is not assured c) For children in countries with limited access to alternative pediatric treatment	a. Pretreatment drug resistance to determine the prevalence of NNRTI resistance in adults b. HIVDR surveillance in children <18 months	PDR in patients more at risk of having NNRTI resistance or in all patients based on the prevalence of NNRTI resistance in countries not able to switch all patients to non-NNRTI based regimen	-Accelerate transition to dolutegravir based first-line regimen -Assess cost-effectiveness of switching to dolutegravir -based first-line in countries without access to generic low-cost dolutegravir -Accelerate transition to non-NNRTI-based pediatric treatment and in women of reproductive age who are not able to access effective and reliable contraceptives
bPI-resistance	Need to preserve treatment options	-PDR survey in patients initiating or re-initiating bPI-based ART -Acquired drug resistance in patients on bPI based regimens	Patients with virological failure, to prevent unnecessary switches and optimize next-line of treatment	-Determine optimum sequencing strategies, preserve treatment options and optimize third-line regimen

ART=antiretroviral therapy; bPI=ritonavir boosted protease inhibitors; DRT= drug resistance test; HIVDR= HIV drug resistance; LMICs = low- and middle-income countries; NRTI= nucleoside reverse transcriptase inhibitors; NNRTI= non-NRTI; PDR=pretreatment drug resistance; PrEP=pre exposure prophylaxis

Access to affordable viral load and affordable HIV drug resistance testing

To support the proposed monitoring strategies and maximize the gains of DTG-based regimen, there is need to support current efforts for universal access to routine viral-load tests. Strategies to improve viral load testing have previously been reviewed, with a strong emphasis on using point-of-care tests for increasing decentralized access, use of dried blood spots specimens, creating demand by increasing treatment literacy among the communities and addressing gaps in the viral load testing cascade to ensure efficient uses of resources.[62,63]

Equally, the need for HIV drug resistance tests for both individualized patient management and population-based surveillance will increase during the dolutegravir era. A number of HIVDR genotyping technologies are becoming increasingly affordable, as reviewed elsewhere.[64,65] Increased political will and investments are needed to actualize affordable HIVDR testing in LMICs.

Conclusion and future directions

As the therapeutic landscape for ART in LMICs changes dramatically, with potential for more efficacious and durable therapy based on DTG, a similar transition needs to be made to improve on the monitoring framework to ensure sustained optimal treatment outcomes. There is a paucity of data on DTG resistance in the context of the WHO public health approach to ART, with limited access to virological monitoring and among circulating non-B subtypes. We caution that focusing on medical-technical solutions alone risks complacency, whilst curbing resistance requires a multi-faceted approach. There is an urgent need for implementing a framework for the systematic and standardized monitoring of patients on DTG-based treatment, in order to determine new mutation patterns not previously observed or well-understood as well as the magnitude of DTG-associated resistance development in LMIC.

Authors' contributions

SCI, RLH, TFRW conceptualized the paper. SCI drafted the manuscript, with assistance from RLH and TFRW. All authors reviewed and contributed to subsequent drafts for important intellectual content, and approved the final manuscript. The views expressed in this review are those of the authors and may not necessarily reflect those of the institutions for which they work.

Conflicts of interest

The authors declare no competing interests.

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Part II

Burden of PDR among HIV-infected infants

Chapter 9

High prevalence of HIV drug resistance among newly diagnosed infants aged <18 months: results from a nationwide surveillance in Nigeria

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ABSTRACT

Background

WHO recommends protease-inhibitor-based first-line regimen in infants because of risk of drug resistance from failed prophylaxis used in prevention of mother-to-child transmission (PMTCT). However, cost and logistics impede implementation in sub-Saharan Africa, and >75% of children still receive nonnucleoside reverse transcriptase inhibitor-based regimen (NNRTI) used in PMTCT.

Methods

We assessed the national pretreatment drug resistance prevalence of HIV-infected children aged <18 months in Nigeria, using WHO-recommended HIV drug resistance surveillance protocol. We used remnant dried blood spots collected between June 2014 and July 2015 from 15 early infant diagnosis facilities spread across all the 6 geopolitical regions of Nigeria. Sampling was through a probability proportional-to-size approach. HIV drug resistance was determined by population-based sequencing.

Results

Overall, in 48% of infants (205 of 430) drug resistance mutations (DRM) were detected, conferring resistance to predominantly NNRTIs (45%). NRTI and multiclass NRTI/NNRTI resistance were present at 22% and 20%, respectively, while resistance to protease inhibitors was at 2%. Among 204 infants with exposure to drugs for PMTCT, 57% had DRMs, conferring NNRTI resistance in 54% and multiclass NRTI/NNRTI resistance in 29%. DRMs were also detected in 34% of 132 PMTCT unexposed infants.

Conclusion

A high frequency of PDR, mainly NNRTI-associated, was observed in a nationwide surveillance among newly diagnosed HIV-infected children in Nigeria. PDR prevalence was equally high in PMTCT-unexposed infants. Our results support the use of protease inhibitor-based first-line regimens in HIV-infected young children regardless of PMTCT history and underscore the need to accelerate implementation of the newly disseminated guideline in Nigeria.

INTRODUCTION

Interventions to prevent mother to child HIV transmission (PMTCT) include providing combination antiretroviral therapy (cART) for pregnant HIV-positive women and nevirapine and/or zidovudine prophylaxis for HIV-exposed infants[1]. Despite the successful global scale-up of PMTCT, over 150,000 infants worldwide still become infected each year[2]. It is estimated that over 27% of these pediatric infections occur in Nigeria (~41,000 newborns)[2]. The overall HIV epidemic in Nigeria is estimated at 3.4 million people, of whom ~270, 000 are children (<15 years)[3]: this represents the second-largest HIV epidemic globally including 15% of all HIV-positive children[4]. Nigeria has also the second-largest burden of new infections in women worldwide and one of the highest mother-to-child transmission rates estimated at 23%[2]. ART coverage in the country is far from optimal: an estimated 2 out of 3 pregnant women do not receive antiretroviral therapy and ART coverage amongst children is even lower (in the 14-17% range)[2]. Out of the global pediatric HIV death rate, Nigeria represents 23% (35,000 children/year)[4].

Since 2013, WHO recommends the use of protease inhibitors (PI) in first-line ART for HIV-infected children below 3 years of age in resource-limited countries[5]. Due to financial and logistical constraints, PIs are however often reserved for those young children who have been exposed to PMTCT[6,7]. Children without PMTCT exposure, still often receive non-nucleoside reverse transcriptase inhibitors (NNRTI)-based ART. A previous study describing pretreatment drug resistance among children <12 years in Lagos, Nigeria, showed that PDR was also higher among children with no recorded exposure to PMTCT drugs, being present in 1 out of 6 cases[8]. The expected effect on the outcomes of first-line ART were proven detrimental as the presence of pretreatment drug resistance appeared the strongest predictor of pediatric therapy failure, as also demonstrated in a parallel study in Uganda[8,9]. As a follow-up to this study and as recommended by WHO, we further conducted a nationwide survey to determine the national prevalence of pretreatment drug resistance (PDR) among young infants newly diagnosed with HIV in Nigeria.

METHODS

Study design

We performed a nationwide, laboratory-based, cross-sectional assessment of pretreatment HIV-1 drug resistance among infants aged <18 months from remnant dried blood spots (DBS) collected in Nigeria through the national early infant diagnosis (EID) program between June 2014 and July 2015. The study protocol was based on the WHO generic protocol for "surveillance of initial drug resistant HIV-1 among children <18 months of age newly diagnosed with HIV"[10].

Samples were obtained from 15 EID facilities spread across all the geopolitical regions. Nigeria is a federal republic with 36 states and one federal capital territory. The states are further grouped into six geopolitical zones based on geopolitical considerations; North East, North West, North Central, South West, South East and South-South (Figure 1). The number of samples contributed per laboratory was based on the size of the facility, using a probability-proportional-to size approach. The size of each laboratory was determined by the number of HIV-positive diagnoses in a given time period. The proportion of positive test per laboratory determined the proportion of samples contributed to the HIV drug resistance survey. The total sample size was N= 481 approximated to 500, based on a predicted prevalence of HIV drug resistance of 50% and a non-amplification rate of 20%. This sample size was later increased to 549 after resampling due to non-response from some sites, poorly collected DBS and amplification failures.

The study received a non-research determination approval from the National Health Research Ethics Committee, Nigeria as a routine surveillance study.

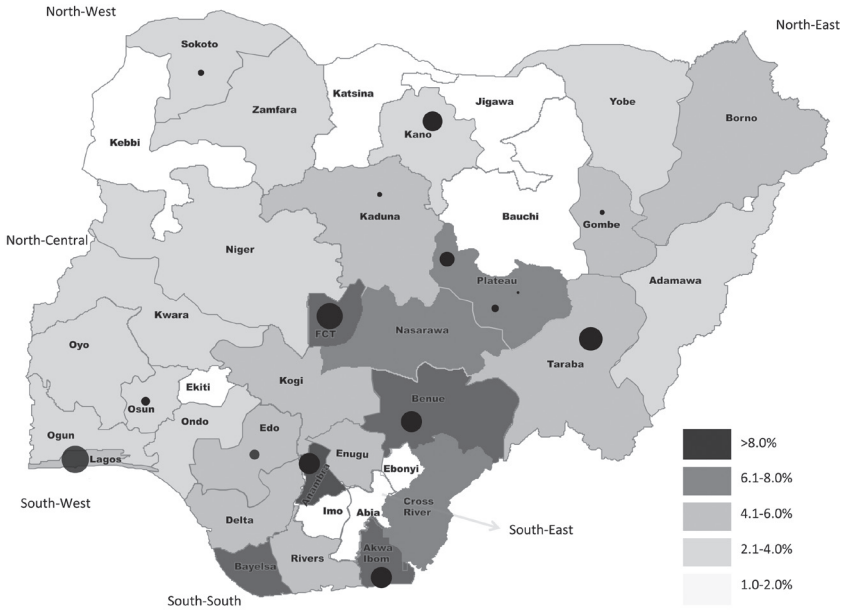

Figure 1. Geographical map of Nigeria indicating the EID laboratories within the 6 geopolitical zones that contributed samples to the survey.

States within the North-East region; Adamawa, Bauchi, Borno, Gombe, Taraba, Yobe. North-West region; Jigawa, Kaduna, Kano, Katsina, Kebbi, Sokoto, Zamfara. North-Central region; Benue, Kogi, Kwara, Nasarawa, Niger, Plateau, Federal Capital Territory (FTC). South-East region; Abia, Anambra, Ebonyi, Enugu, Imo. South-South region; Akwa Ibom, Cross River, Bayelsa, Rivers, Delta, Edo. South West region; Ekiti, Lagos, Ogun, Ondo, Osun, Oyo. The circles indicate an EID laboratory facility that contributed specimens to the survey with the size proportional to the number of specimens. The grey scale indicates the HIV prevalence rate in each state.

Data collection, sample handling and sequencing procedure

Clinical data was collected from standard EID lab request forms and entered into an electronic database in the coordinating site at Lagos university teaching hospital (LUTH). Collected DBS samples were sent to LUTH for combined shipment to the WHO-accredited KEMRI/CDC HIV drug resistance regional reference laboratory in Kisumu, Kenya. At LUTH, the sample IDs on the request forms and DBS were verified before storage at -20°C. Prior shipping, fresh silica-gel desiccant sachets were added into the zip-locked bags together with humidity indicators. Specimens were then shipped at room temperature and stored at -20°C upon arrival at KEMRI/CDC.

Pol gene sequences were obtained using the CDC in-house Sanger sequencing assay[11]. Briefly total nucleic acid was extracted from DBS using NucliSense

(bioMerieux, Inc., Durham, NC) but with enhanced lysis using reagents and procedures from QiaAmp DNA blood mini kit (Qiagen Inc, Chatsworth, CA). About 1080-base pair segment of the 5' region of the pol gene was then generated by a two-step RT and nested PCR. This fragment was then purified, sequenced using the BigDye® Terminator v3.1 Cycle Sequencing Kit (Applied Biosystems, Foster City, CA), and analyzed on the ABI Prism™ 3130 Genetic Analyzer (Applied Biosystems, Foster City, CA). Sequence data was assembled with RECall (University of British Columbia, Canada) and quality assessed as per WHO recommendations, using RECall, molecular evolutionary genetic analysis (MEGA v7.0) tool and Stanford HIVdb tools. Resistance was assessed using the Stanford HIVdb algorithm (v 7.0) and subtyping by Rega HIV subtyping tool (v 2.0). Descriptive statistics were performed using Stata v12.0.

RESULTS

Twenty EID facilities in Nigeria were approached to participate in this survey, according to WHO recommendations. Samples for 549 Nigerian infants from 15 EID facilities were collected and included in the study (Figure 1). Five facilities (2 from South-West, 1 each from North-East, North-Central and South-East regions) did not participate in the study, either due to late or poor communication, logistical challenges or lack of viable samples. To correct for this, more samples were collected from the other EID labs with re-distribution done within the geo-political regions. Under the adjusted sampling, the South-South region, was under-represented by 7% (Table 1).

Drug resistance results were available for 430 (78%) children. Missing genotypic results were due to failure to amplify in 99 samples and quality assurance issues in 20 samples (APOBEC mutations n=5, poor quality sequence n=1, unresolved close genetic distance n=6, duplicate samples n=8).

The median age of participating newborns was 5.1 months (IQR 2.0-9.0); there was a near equal distribution between boys and girls (46% vs 44%, missing data 10%). Exposure to PMTCT drugs, either to maternal or infant regimen was reported in 47% (204) of children while 31% (134) were unexposed. Of the 204 infants with PMTCT exposure, only 72% (146) had complete information on exposures to both neonatal and maternal regimens; 71% (104) had been exposed to both

maternal and neonatal PMTCT, 12% (18) to only maternal and 16% (24) to only neonatal regimens. Data on previous exposure to PMTCT drugs was however missing in 21% (92) of children.

Neonatal PMTCT prophylaxis consisted of single dose nevirapine in 14% (59), extended prophylaxis in 19% (81), was not administered in 27% (118) and missing in 40% (172) of children. Information on maternal use of antiretroviral drugs for PMTCT was missing in 26% (112) of infants. If maternal PMTCT was reported (318), it consisted of a triple regimen in 37% (118), and of mono- or dual-therapy in 7.5% (24), unknown regimen in 7.8% (25) and not administered in 47% (151). Seventy-eight percent (334) of children had ever breast-fed or were breast-feeding at the time of sample collection.

Prevalence of pre-treatment drug resistance

In 48% of infants (n=205 of 430), drug resistance mutations (DRM) were detected, conferring resistance to predominantly NNRTIs (45.1%, 194). Only 22% (94) of the infants had NRTI DRMs. Multi-class resistance to both NRTI and NNRTI drugs was present in 20% (88), and 2% (7) had protease inhibitor associated DRMs.

Table 1 further shows the PDR prevalence by geo-political region. The prevalence varied by site (p=0.007) being highest in South-East and North West and lowest in South-West region but these differences did not correlate with PMTCT coverage (p=0.614).

Table 1: Prevalence of pretreatment drug resistance amongst HIV-1 infected infants across all geo-political regions in Nigeria

Geo-political region	*PMTCT coverage%	No of EID facilities†	Assigned samples (n)	Available sequences (n)	Any DRM (n)	Any DRM (%, 95%CI)	Any NNRTI DRM (n)	Any NNRTI DRM (%, 95%CI)
North-Central	46.3	5	188	145	78	54 (46-60)	74	51 (40-59)
North-East	15.3	2	37	53	24	45 (32-60)	23	43 (30-57)
North-West	10.0	3	48	45	26	58 (43-70)	25	56 (40-70)
South-West	19.2	2	45	90	29	32 (22-40)	27	30 (20-40)
South-South	42.5	1	73	51	21	41 (27-50)	20	39 (30-53)
South-East	57.2	2	29	46	27	59 (44-70)	25	54 (40-69)

Geo-political region	Any NRTI DRM (n)	Any NRTI DRM (%, 95%CI)	Any RTI DRM (n)	Any RTI DRM (%,95%CI)	Any PI DRM n)	Any PI DRM (%, 95%CI)	Prevalent Sub-type (%)
North-Central	38	26 (20-33)	36	25 (18-30)	3	2 (0-4)	G (43)
North-East	11	21 (10-32)	10	19 (8-30)	0	0	G (51)
North-West	16	36 (20-50)	15	33 (19-50)	1	2 (0-7)	G (62)
South-West	10	11 (5-18)	9	10 (4-16)	1	1 (0-3)	CRF_02AG (47)
South-South	4	8 (0-15)	3	6 (0-12)	0	0	CRF_02AG (49)
South-East	15	33 (20-46)	15	33 (19-46)	2	4 (0-10)	G (52)

* Average PMTCT coverage based on 2014 estimates (http://naca.gov.ng/2014-annual-report-on-health-sector-hiv-and-aids-in-nigeria/)
†Five facilities did not participate, 2 in south-west,1 each in North-East, North-central & South East

Forty-five percent (194 of 430) of the infants had intermediate to high-level resistance to nevirapine, 45% (193) to Efavirenz and 20% (86) and 18% (78 of 430) to the second-generation NNRTI's rilpivirine and etravirine respectively. The most common NNRTI mutations were K103N and Y181C constituting 59% (120) and 31% (64) respectively of the 205 children with PDR (Figure 2).

For the NRTI's, intermediate to high-level resistance was highest for lamivudine and emtricitabine (16%, 68 of 430), due to a predominance of M184V mutation (72%, 68 of 205). Resistance to abacavir and zidovudine was each at 7%, (n=30 and 28 respectively), and lowest for tenofovir (3%, 11). Overall 6% (27) and 5% (22) of the children were resistant to all drugs in the commonly used NNRTI based combinations of zidovudine/nevirapine/lamivudine and abacavir/lamivudine/nevirapine respectively.

Resistance to PIs was only at potential low-level, and comprised of M46L (2.4%, 5) and G173S (0.5%, 1) (Figure 2).

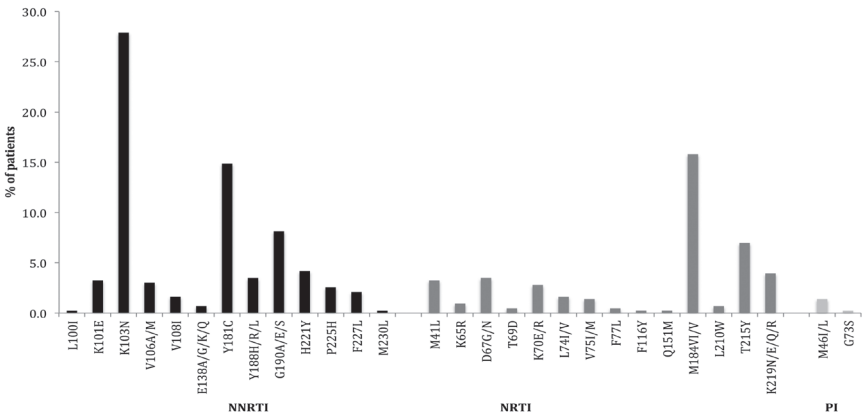

Figure 2. Drug-resistance mutation patterns in HIV-infected infants in Nigeria aged <18 months.

NNRTI, non-nucleoside reverse transcriptase inhibitor; NRTI, nucleoside reverse transcriptase inhibitor; RT, dual NRTI and NNRTI resistance; PI, protease inhibitor.

PDR and PMTCT exposure

Among 204 infants with exposure to drugs for PMTCT, 57 % (116) had any DRMs, 54% (111) had DRMs conferring resistance to the NNRTI, 30% (62) to NRTIs, 29% (60) to multi-class NRTI/NNRTI and 2% (5) to PIs (Figure 3).

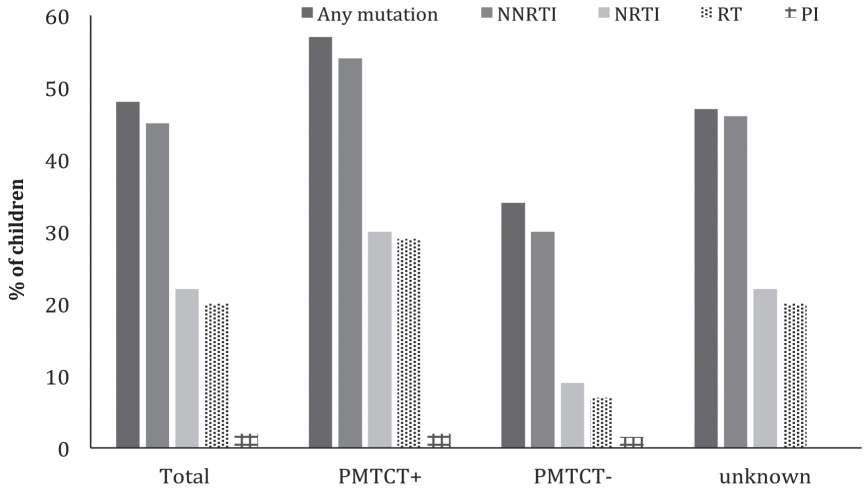

Figure 3: Drug-resistance patterns based on exposure to drugs for the prevention of mother-to-child trans- mission (PMTCT) in HIV-infected infants in Nigeria aged <18 months.

NNRTI, non-nucleoside reverse transcriptase inhibitor; NRTI, nucleoside reverse transcriptase inhibitor; RT, dual NRTI and NNRTI resistance; PI, protease inhibitor.

Exposure to both maternal and neonatal prophylaxis resulted in higher levels of PDR (61%, 63/104) compared to exposures to only maternal prophylaxis (39%, 7/18 p=0.09) or neonatal prophylaxis (29%, 7 of 24 p=0.015). NRTI and multi-class NRTI/NNRTI resistance were also more frequent among infants exposed to both maternal and neonatal ART compared to those with neonatal prophylaxis exposure only (NRTI 36% vs 8% p=0.012, multi-class NRTI/NNRTI 35% vs 4% p=0.007) and borderline significance to those exposed to only maternal ART (NRTI 11%, p=0.054 and multi-class NRTI/NNRTI 11%, p=0.053).

Among 134 PMTCT unexposed infants, DRMs were detected in 34% (45/134) of infants (Figure 3). In the unexposed infants most mutations were also NNRTI-associated (30%, 40). NRTI resistance was present in only 9% (12), multi-class NRTI/NNRTI resistance was present in 7% (9) and 2% (2) had PI resistance.

Among infants with missing information on ART exposure, DRM was detected in 48% (44/92), NNRTI in 47% (43), NRTI in 22% (20) and multi-class NNRTI/NRTI resistance in 21%. (19).

Sub-type distribution

The most predominant subtypes were G (45%, 194) and circulating recombinant form (CRF), CRF02_AG recombinant (38%, 163). Subtype G predominated in the northern regions and South-East regions while CRF02_AG in South-West & South-South regions. Other majority subtypes included A (5%, 21) and recombinants of either G or CRF02_AG (4%, 9). None-G or AG recombinants included 2 subtypes B (0.5%) from North-Central, 2 C's (0.5%) in North-East and North-West, and 2 D's (0.5%) from North-Central and South-West.

DISCUSSION

In this nationwide study, we report that nearly 48% of newly diagnosed HIV-infected infants between 0 and 18 months of age in Nigeria have pretreatment drug-resistance. The majority of these infants harbor resistance against NNRTI-based ART regimens but in addition, about 20% have multi-drug class resistance mutations to both NNRTI and NRTI. The inference from this findings is that although rollout of PMTCT has widely resulted in significant declines of MTCT[2], the chance of harboring HIV drug resistance is alarmingly high for those children who do get infected [12]. Moreover, the prevalence of PDR was also high among PMTCT unexposed infants. These observations are in line with what has been reported in other surveys conducted in sub-Saharan Africa including a 60% prevalence in Togo[13], 63% in both Zimbabwe and South Africa[14,15], and 35% in Swaziland[15]. With an estimated 120,000 new pediatric infections in sub-Saharan Africa, it can be projected that about half of these infants will be having pre-treatment HIV drug resistance, potentially leading to poor health and low life expectancy in absence of more potent regimens.

To address HIV drug resistance in children below 3 years of age, WHO guidelines recommend the use of protease inhibitors as first-line therapy[1]. As with some other countries in sub-Saharan Africa, the adoption of this strategy in Nigeria is limited to children with PMTCT exposures due to cost and logistics constraints[7]. The current study indicates however, that a high HIVDR prevalence still occurs in the absence of reported PMTCT exposure. This is in line with findings in other African countries, like Swaziland, South-Africa and Togo [12,13,15,16]. The high-levels of PDR prevalence in PMTCT-unexposed children in this study may however be over-estimated, citing a low prevalence of transmitted drug resistance among

pregnant women in Nigeria[17–19]. It is thus likely that some of these children could have been exposed but were misclassified possibly due to poor-record keeping, poor recall from the mother or caretaker, or uncertainties in this information in case of maternal deaths prior to disclosures. Nonetheless, this alarming high PDR prevalence, especially from a country with the second-highest HIV burden globally including the highest numbers of HIV-infected children, continue to highlight the universal implementation of the revised WHO guidelines recommending the use of PI-based first-line treatment for all infants regardless of PMTCT exposure. While initial access to PI-based formulations had been greatly hindered by poor palatability and refrigeration requirement, the recent approval of the heat-stable lopinavir-ritonavir pellets overcomes this challenge and may likely lead to increased access in these settings[20]. However high prices of the pellets (508.8 USD per year compared to 84 USD for the low-cost NNRTI; AZT/3TC/NVP) may still hinder their wide-accessibility in resource-limited settings, suggesting an increased need for cost-reductions[21,22]. Notwithstanding, ART programs in sub-Saharan Africa should endeavor to avail these pellets citing the increasing reports of high PDR levels in children. Moreover attempts should also be made to actively monitor infants initially placed on NNRTI-based regimens for timely switching to the PI-based formulation in cases of treatment failure.

As observed in this and other studies, was the high level of pre-treatment NRTI resistance, which was linked to exposure to maternal ART. This is also worrisome due to the potential impact on the current recommended second-line option in young children upon failure to the PI-based 1st line therapy. In the absence of wide-options of pediatric ART regimens, WHO recommends raltegravir plus NRTIs as the preferred regimen for children <3 years[1], but this is likely to be suboptimal based on findings from the SWITCHMRK study[23]. It is also likely that pre-treatment NRTI resistance may increase as countries widely adopt the recommended option B+ PMTCT strategy[24,25]. The increase of pre-treatment NRTI resistance may effectively imply only one potent option of drugs for children <3 years of age under the current pediatric ART landscape. Henceforth efforts are needed to increase pediatric treatment options. Moreover due to the aforementioned challenges in pediatric HIV management in sub-Saharan Africa, there is need for efforts to prevent the occurrence of PDR in infants. This could include adapting more optimal PMTCT strategies similar to those used in western countries, in particular, more frequent monitoring of maternal viral-load during gestation and breast-feeding to enhance prevention of HIV transmission

or in case of PMTCT failure, resistant strains. Those having high viremia could be switched to integrase inhibitors so as to facilitate rapid viral suppression[26]. This could become increasingly feasible with the rollout of the low-cost generic dolutegravir as emerging data also indicate its better safety profile during pregnancy[27]. In addition, resistance acquired from exposure to the sub-therapeutic drugs given as prophylaxis, or from sub-optimal doses of maternal antiretroviral treatment ingested during breast-feeding, could be prevented by use of triple ART prophylaxis.

PDR prevalence varied across the different geographical zones but the pattern did not correspond with PMTCT coverage. The lack of the expected correlation, could possibly be explained in part by the quality of antiretroviral service delivery coupled with sampling bias in our study. Notably there have been concerted efforts by PEPFAR to improve antiretroviral service delivery in the South-West region which may partly explain the low levels of PDR in this region. This observation continues to highlight the need for not only improved access to the prophylactic regimens, but also strengthening the quality of services to ensure effectiveness of PMTCT programs.

Study limitations exist. First there were practical constraints that affected the sampling process. According to the survey methodology, all the EID facilities, 23 in number were required to participate in generating an estimate of the national PDR prevalence. This appeared in practice an important challenge, which will be described elsewhere. In short, there were issues with respect to actual quality of the DBS samples (insufficient numbers and sizes of spots), problems with labeling and completeness of data collection, issues around participation in the survey for some EID sites, challenges due to the necessity to export the samples to Kenya for HIVDR testing, etc. We also experienced a moderately low amplification rate attributed predominantly to the insufficient number and size of spots but also possibly low sample integrity caused by poor storage conditions in some of the EID laboratories. The reduced participation rate and relatively high numbers of invalid samples led to the need to resample so as to improve the power of the study but even with this, it appeared not possible to fully reach the ideal sampling approach as recommended by WHO in its PPS procedure[10]. This could have led to a biased estimate of the national PDR prevalence. Moreover, due to data incompleteness on maternal and infant prophylaxis, it was not feasible to accurately estimate the potential source of PDR. Lastly, we used population-

based Sanger sequencing which might have under-estimated the prevalence of minority variants due to a relatively low-sensitivity associated with this type of assays.

In conclusion, the success of PMTCT is without doubt and numbers of HIV infections in young infants have decreased significantly over the past years. However, in those circumstances that PMTCT did not work and young children do get HIV-infected, a significant majority of them will harbor PDR, which will negatively determine future morbidity and mortality in the absence of optimum regimens. In addition, our findings showing a high PDR in PMTCT unexposed infants, indicates the inadequacy of PMTCT history as a proxy measure for ruling-out PDR and guiding the choice of treatment for young children.

All in all, the findings presented in this study underscore the need to accelerate implementation of PI-based first-line regimens for all infants in this population. This implies urgently addressing the financial and logistical barriers to wide accessibility of PI-based pediatric treatment. Furthermore, given the high levels of NRTI and multi-class NRTI/NNRTI resistance, there is need for prevention of PDR as well as evaluation of alternative treatment options for infants < 3 years in case of failure to PI-based first-line regimens.

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Chapter 10

Prevalence and dynamics of the K65R drug resistance mutation in HIV-1-infected infants exposed to maternal therapy with lamivudine, zidovudine and either nevirapine or nelfinavir in breast milk

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ABSTRACT

Background

K65R is a relatively rare drug resistance mutation (DRM) selected by the NRTIs tenofovir, didanosine, abacavir and stavudine and confers cross-resistance to all NRTIs except zidovudine. Selection by other NRTIs is uncommon.

Objectives

In this study we investigated the frequency of emergence of the K65R mutation and factors associated with it in HIV-1-infected infants exposed to low doses of maternal lamivudine, zidovudine and either nevirapine or nelfinavir ingested through breast milk, using specimens collected from the Kisumu Breastfeeding Study.

Methods

Plasma specimens with viral load ≥1000 copies/mL collected from HIV-infected infants at 0–1, 2, 6, 14, 24 and 36 weeks of age and maternal samples at delivery were tested for HIV drug resistance using Sanger sequencing of the polymerase gene. Factors associated with K65R emergence were assessed using Fisher's exact test and the Wilcoxon rank-sum test.

Results

K65R was detected in samples from 6 of the 24 infants (25%) who acquired HIV-1 infection by the age of 6 months. K65R emerged in half of the infants by 6 weeks and in the rest by 14 weeks of age. None of the mothers at delivery or the infants with a positive genotype at first time of positivity had the K65R mutation. Infants with K65R had low baseline CD4 cell counts (P=0.014), were more likely to have DRMs earlier (≤6 weeks versus ≥14 weeks, P=0.007) and were more likely to have multiclass drug resistance (P=0.035). M184V was the most common mutation associated with K65R emergence. K65R had reverted by 3 months after cessation of breastfeeding.

Conclusions

A high rate of K65R emergence may suggest that ingesting low doses of lamivudine via breast milk could select for this mutation. The presence of this mutation may have a negative impact on future responses to NRTI-based ART. More *in vitro* studies are, however, needed to establish the molecular mechanism for this selection.

INTRODUCTION

The introduction of antiretroviral chemoprophylaxis for prevention of mother-to-child transmission (PMTCT) has led to significant reductions in paediatric HIV acquisition.[1,2] Despite this success, a significant number of infections still occur; in 2014, ~220000 paediatric infections occurred worldwide with 99% in sub-Saharan Africa.[1] Subsequent care, treatment and survival of the infected infants are issues of concern, with treatment success being hampered by drug resistance.[3–12] Previous studies have documented the emergence of both acquired and transmitted drug resistance variants in infants.[3,4,7–9,11] Acquired drug resistance occurs due to incomplete suppression of viral replication during administration of antiretroviral drugs to infants or by ingestion of maternal drugs through breast milk. The latter has been shown to result in drug resistance mutation (DRM) patterns including multidrug resistance, defined as resistance to more than one drug within NRTIs or NNRTIs, and multiclass resistance, defined as resistance to two or more classes of antiretroviral drugs, such as resistance to NRTIs and NNRTIs.[3] Emergence of multidrug resistance and multiclass resistance has significant implications as they could limit future treatment options for the HIV-infected infants.

One multidrug resistance mutation of concern is K65R as it causes the loss of drug susceptibility of HIV-1 to all NRTIs with the exception of zidovudine.[13–17] The K65R mutation is usually selected by tenofovir disoproxil fumarate, didanosine, abacavir and, to a lesser extent, stavudine usage.[14,16,18] Its occurrence is relatively infrequent, but there has been a significant rise with increased use of tenofovir.[16,17] Little information exists on selection of K65R with lamivudine usage, while the use of zidovudine has been shown to inhibit its emergence.[16,19]

In the present study, we investigated the frequency of K65R emergence and factors associated with it in HIV-infected infants exposed to low doses of zidovudine, lamivudine and either nevirapine or nelfinavir via breast milk in the Kisumu Breastfeeding Study (KiBS) conducted in 2003–09.

METHODS

Study participants

KiBS was a phase IIb PMTCT single-arm and non-randomized trial that aimed to assess the safety and efficacy of a triple-antiretroviral drug regimen consisting of zidovudine, lamivudine and either nevirapine or nelfinavir from 34-week gestation through 6 months post-partum for PMTCT among HIV infected breastfeeding mothers. Single dose nevirapine was administered to infants within 72 hours of birth. Details of the study findings have previously been described.[20] Both mothers and infants were followed for up to 18 months after cessation of breastfeeding. The current analysis focuses on up to 9 months post-partum. Infants were initiated on antiretroviral therapy based on CD4 cell counts with the regimen being determined by a clinical team, as there were no standard guidelines for antiretroviral therapy for infants in Kenya at that time.

Specimen collection and laboratory testing

Whole blood samples were collected from the infants in EDTA-treated anti-coagulant Vacutainers (Becton Dickinson, Franklin Lakes, NJ, USA). Haematological and virological laboratory tests were performed at 0–1, 2, 6, 14, 24 and 36 weeks of age. Roche DNA-PCR, version 1.5 (Roche Diagnostics Systems, Branchburg, NJ, USA) was used to diagnose HIV-1 infections in infants at 14, 24 and 36 weeks with dried blood-spot specimens. For those infants who were HIV-1 DNA PCR positive using dried blood spots, PCR was performed retrospectively on previously collected specimens to determine the timing of HIV infection. CD4 cell counts and viral load were assessed during multiple study visits using FACSCalibur (Becton Dickinson, Franklin Lakes, NJ, USA) and the Roche Amplicor HIV-1 Monitor Test, v 1.5 (Roche Diagnostics Systems, Branchburg, NJ, USA), respectively.

HIV-1 genotyping and viral subtyping

HIV drug resistance genotyping was conducted retrospectively on plasma specimens collected from infants with viral loads of ≥1000 copies/mL at 14 weeks of age, using the ViroSeq™ HIV-1 Genotyping System (Applied Biosystems, Foster City, CA, USA). For those infants with detectable DRMs at 14 weeks of age, progressively earlier samples (6, 2 and 0–1 weeks) were then analysed to determine the time when the HIV DRM first emerged. In addition, drug resistance testing was conducted on all infant samples collected at 24 and 36 weeks of age by use of either ViroSeq or an in-house assay previously described.[21]

Analysis of DRMs was performed with the ViroSeq HIV-1 genotyping analysis software, version 2.6, and with the Stanford HIVDB algorithm (Stanford University, CA, USA) together with the International AIDS Society-USA mutation list-2011 update.[22,23] To determine the extent of genetic diversity, sequences obtained were aligned using the Se-Al 1.0 multiple sequence alignment program, and subjected to phylogenetic analysis using parsimony (PAUP*) 4.0b software along with HIV-1 reference sequences from an HIV database (Los Alamos database). HIV-1 inter-subtype recombination was analysed using Simplot software, version 3.5.1.[24]

Statistical analysis

Fisher's exact test was used to compare categorical variables and the Wilcoxon rank-sum test was used to compare continuous variables to determine factors associated with K65R emergence.

Ethics statement

This study was approved by the Ethical Review Committee of the Kenyan Medical Research Institute (KEMRI), as well as the Institutional Review Board of the US CDC, Atlanta, GA, USA. All mothers provided written informed consent that included parental consent for their infants.

RESULTS

Based on the previously published data[3,20] a total of 32 infants acquired HIV-1 during the study period, 24 (75%) of whom were infected by the age of 6 months. Among the 24 infants, 16 developed DRMs during the 6 month breastfeeding period. Table 1shows that among the 16 infants that had DRMs detected, 17% and 13% already had multidrug resistance mutations and multiclass resistance mutations at the first time of DRM emergence, respectively. The K65R mutation was detected in 6 (25%) of the 24 infants who acquired HIV-1 infection by the age of 6 months; these 6 were among the 16/24 who had detectable DRMs (6/16, 37.5%). Of these 16 infants, 10 had a genotype at the first time of positivity and none had K65R mutation. Of the remaining six, only two had the K65R mutation at the next earliest timepoint (Table 1). Most infants had documented HIV-1 infection by the age of 2 weeks, and the median time of K65R detection from these infants was at the age of 14 weeks (Table 1). In addition, the K65R mutation

was only detected from specimens collected during the breastfeeding period, at which time none of the infants was on ART for their own treatment.

Table 1. Therapeutic and virologic characteristics of HIV-1 infected infants who developed drug resistance mutations during six months of breastfeeding period, KiBS Study 2003-2009, Kisumu, Kenya

Infant #	Mother's Regimen	Time of first positive HIV-PCR	HIV RNA level at first positivity (log10 copies/mL)	Mutation at first positivity	Time of Resistant Emergence	Mutations at Emergencec
1-0457-9[a]	NFV/ZDV/3TC	0-1wk	5.26	K65R+ M184Vb	6wk	K65R+ M184V
1-0496-6[a]	NFV/ZDV/3TC	0-1wk	5.48	K65R+ M184Vb	6wk	K65R+ M184V
1-0472-8[a]	NVP/ZDV/3TC	0-1wk	3.48	WT	14wk	K65R+M184V+ K103N+Y181C
1-0079-3[a]	NVP/ZDV/3TC	0-1wk	4.73	WT	6wk	Y181C+G190A
1-0066-8[a]	NVP/ZDV/3TC	0-1wk	4.42	WT	14wk	K65R + G190A
1-0410-4[a]	NFV/ZDV/3TC	6wk	4.64	M184I/V	6wk	M184I/V
1-0195-6	NVP/ZDV/3TC	0-1wk	2.11	WT	6mo	K103KN
1-0212-2	NVP/ZDV/3TC	6mo	5.42	Y181C	6mo	Y181C
1-0230-2	NVP/ZDV/3TC	0-1wk	4.66	WT	14wk	M184V
1-0317-8	NVP/ZDV/3TC	14wk	3.76	M184I+Y181C	14wk	M184I+Y181C
1-0289-1	NFV/ZDV/3TC	2wk	3.87	WT[b]	14wk	M184V
1-0357-5	NFV/ZDV/3TC	0-1wk	4.01	M184V[b]	14wk	M184V
1-0360-0	NFV/ZDV/3TC	0-1wk	6.35	M184V[b]	6wk	M184V
1-0437-5	NFV/ZDV/3TC	14wk	5.30	M184I	14wk	M184I
1-0517-4	NFV/ZDV/3TC	0-1wk	2.75	WT	14wk	M184V
1-0278-8	NFV/ZDV/3TC	0-1wk	3.21	WTb	14wk	M184V

[a] Shows infants who developed K65R mutations; [b] Data at next earliest time point due to undetectable, insufficient sample or failed amplification' [c] Indicates mutations detected at time of first emergence.
KiBS = Kisumu Breastfeeding Study, wk= week, mo= month, NVP= nevirapine, ZDV = zidovudine, 3TC=lamivudine, NFV = nelfinavir,

Patterns of K65R mutation evolution in lamivudine-exposed infants

To study the evolution of the K65R mutation in these infants, we genotyped plasma specimens collected from the 16 infants at 0–1 week and up to 9 months of age (Figure 1). The K65R mutation was first detected in three infants by 6 weeks of age, while it was detected in the remaining infants at 14 weeks of age (Table 2). Of the six infants, four had earlier genotypes, three of which had WT strains and one had only the M184V mutation (Table 2). Three of the mothers had an undetectable viral load at delivery, which was sustained through the breastfeeding period. Of the remaining three, two had sufficient viral load at delivery and yielded a WT genotype.

Table 2: Emergence and fading of resistance mutations among infants with K65R in the KiBS, 2003–09, Kenya

Infant#	Time (wks)	VL (log10)	Resistance	Mo-Vl (log10)	Mo-resistance
1.0066.8[a]	2	4.42	WT	2.74	[c]
	6	5.81	WT	Undetectable	-
	14	3.97	**K65R**, G190A	Undetectable	-
	24	4.25	D67N, T215F, G190A	3.43	[c]
	36		D67N, K70R, M184V, K103N, T215F, G190A, K238T	-	-
1.0079.3[a]	2	4.73	WT	4.90	WT
	6	5.57	Y181C, G190A	2.97	[c]
	14	3.87	**K65R**, M184I/V, K101E, K103N, Y181C, G190A	3.87	[c]
	24	3.94	M184V, K101E, G190A	Undetectable	-
	36	6.15	M184V, K101E, G190A		-
1.0410.4	2	Negative	-	Undetectable	-
	6	4.64	M184I/V		
	14	5.43	**K65R**, M184V	Undetectable	-
	24	5.51	M184V, T215Y	Undetectable	-
	36	5.74	T215D	Undetectable	-
1.0457.9[b]	2	[c]	[c]	Undetectable	-
	6	5.26	**K65R**, M184V	Undetectable	-
	14	5.92	**K65R**, M184V	Undetectable	-
	24	5.92	**K65R**	Undetectable	-
	36	4.35	M184V	-	-
1.0472.8[b]	2	3.48	WT	3.13	WT
	6	5.85	**K65R**, M184V	Undetectable	
	14	5.79	**K65R**, M184V K103N, Y181C	Undetectable	
	24	6.51	**K65R**, M184V, K103N, Y181C	3.84	M184V, K103N
	36	3.37	M184V, K103N, Y181C	-	-
1.0496.8	2	3.26	[c]	Undetectable	
	6	5.48	**K65R**, M184V	Undetectable	
	14	5.99	M184V	Undetectable	
	24	5.79	M184V	4.50	WT
	36	5.30	WT	-	-

The table shows viral load and HIV resistance patterns over the different study visits in the six infants who acquired K65R together with maternal information at corresponding visits. The K65R mutation is highlighted in bold at each timepoint. Four infants started treatment at 6 months. [a]Started NNRTI-based regimen. [b]Started PI-based regimen. [c] Not performed because of insufficient sample or failed amplification.

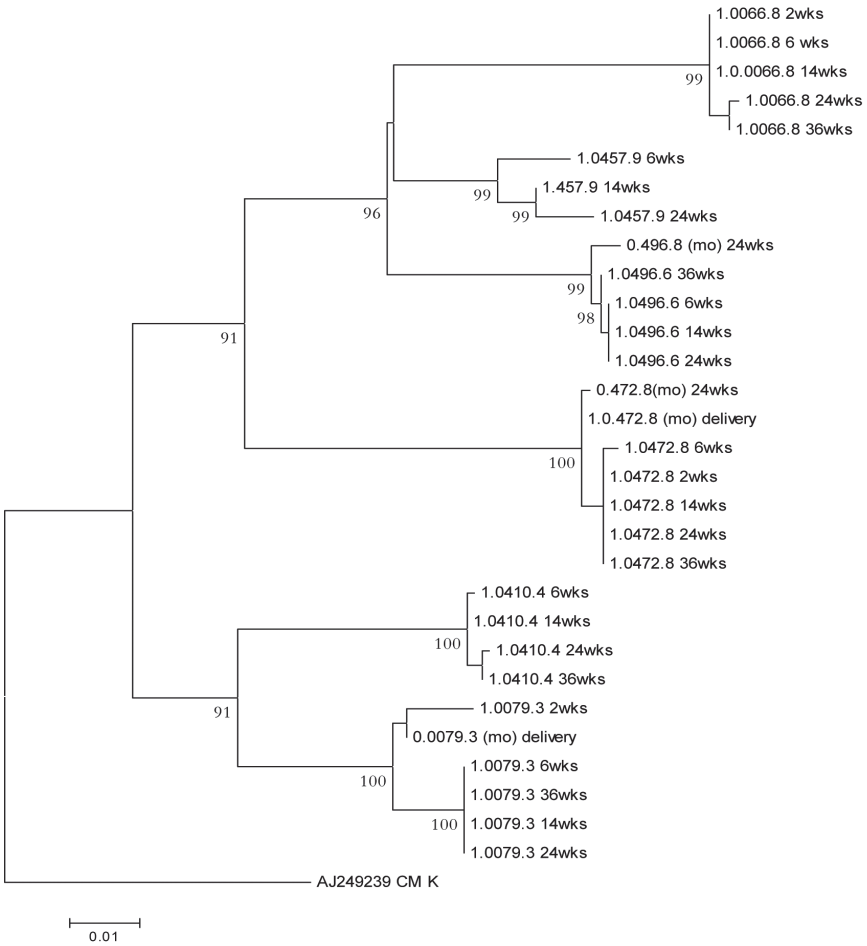

Figure 1: Evolution of infant sequences with K65R.

Phylogenetic tree showing evolutionary relationship of infants' genotypes from first positivity to 9 months. Maternal sequences were also included where possible.

The most common mutation detected in infants with K65R was M184I/V [5 of 6 (83%)]. Some infants also had accompanying NNRTI mutations, which included K101E, K103N, Y181C and G190A. The K65R mutation was undetectable by 6 months of age in four infants and by 9 months in the rest (Table 2). Four of the infants initiated treatment by 6 months [two on PI regimens (lopinavir/ritonavir/abacavir/zidovudine) and two on NNRTI regimens (nevirapine/lamivudine/zidovudine)]. None of these infants had achieved viral suppression by 9 months, but had persisting reverse transcriptase inhibitor mutations with no re-

emergence of the K65R mutation. Sequences analysed in this study have been submitted to GenBank and their accession numbers are HM164112-HM164123, HM164127-HM164128 and HM164130-HM164131.

Factors associated with selection of the K65R mutation

Table 3 summarizes factors associated with K65R mutation, considering all infants who acquired HIV-1 infection by 6 months of age. Infants with K65R were more likely to have developed DRMs by 6 weeks of age compared with those without the mutation (*P*=0.007). They were also likely to have multiclass resistance (*P*=0.035), as well as low baseline CD4 cell counts (*P*=0.014). Maternal regimen (nevirapine based versus nelfinavir based), viral load and CD4 cell counts at delivery and infant's baseline HIV-1 RNA level and timing of infection (before or after 1 week of age) were not significantly associated with K65R mutation emergence (Table 3).

Table 3. Factors associated with K65R mutation in HIV-infected infants participating in the Kisumu Breastfeeding Study, 2003-2009, Kenya

Characteristic	With K65R n=6	Without K65R n=18	P
Maternal VL at delivery, median log_{10} VL copies/mL (IQR)	1.4 (95%CI 0-4.73)	3.4 (2.5-4.4)	0.224
Maternal CD4+ cell count at delivery, median cells/mL (IQR)	290.3 (188.3-664.0)	435 (339.5-526.7)	0.230
Maternal Regimen NVP+ZDV+3TC NFV+ZDV+3TC	 3 (50%) 3 (50%)	 10 (56%) 8 (44%)	 1.0
Infant VL at 1st positivity, median log_{10} VL (IQR)	4.5 (95%CI 3.3-5.2)	5.4 (95%CI 3.9-5.7)	0.205
Infant CD4+ cell count at 1st positivity, median cells/mL (IQR)	1016.4 (95%CI 683.8-1680.7)	1876 (95%CI 1450.4-2073.0)	**0.014**
Time of infection 0-1 wk >1wk-6mo	 5 (83%) 1 (17%)	 7 (39%) 11 (61%)	 0.16
Time of 1st mutation emergence[a] <= 6 weeks >6 weeks	 4 (67%) 2 (33%)	 1 (10%) 9 (90%)	 **0.007**
Multi-class drug resistance[a]	3 (50%)	1 (10%)	**0.035**

wk= week, mo= month, VL=Viral load, NVP= nevirapine, ZDV = zidovudine, 3TC=lamivudine, NFV = nelfinavir, [a] Time of 1st mutation emergence and Multi-class drug resistance was assessed on only the 16 infants who had any resistance, significant p-values >0.05 are highlighted in bold

Subtype distribution

Four (75%) of six infants with the K65R mutation were infected with subtype A viral strains, which were also the predominant variant among all the HIV-1-infected infants in this study. The remaining two infants were infected with an A/D recombinant.

DISCUSSION

We report a high incidence of K65R mutations in infants exposed to sub-optimal concentrations of antiretroviral drugs through breast milk. Our previous study showed the occurrence of DRMs in breastfeeding infants by 6 month of age and illustrated that these mutations were acquired, rather than transmitted, through ingestion of sub-optimal dosages of the maternal regimen in breast milk.[3] None of the infants genotyped at first time of positivity or mothers with a detectable viral load at delivery had the K65R mutation. The mothers of these infants were ART naive at treatment initiation and remained exclusively on the study regimens.[3,20] NRTI mutations were the most commonly detected mutations in these infants. A notable finding was the high incidence of K65R in infants exposed to regimens infrequently known to select for this mutation. We postulated that this mutation could have been selected by lamivudine. In our previous assessment of drug levels in these infants and their mothers we showed that lamivudine and nevirapine were the only drugs detected in infants through ingestion of breast milk in quantities sufficient to have therapeutic effect.[25] In that study we reported drug levels in breast milk and infant's blood at 2, 6, 14 and 24 weeks post-partum; zidovudine was detected at below the quantitative limit (bql) in the infant samples and a low of 9 ng/mL (IQR bql–26) in breast milk. Lamivudine, on the other hand, was detected at high levels in the infant plasma (an estimated daily intake of 182 µg/kg, ~2% of the daily recommendation) and an average of 23 ng/mL (during the assessment period of 2–24 weeks), which is just slightly above the IC_{50} of lamivudine for WT strains (0.6–21 ng/mL).[25,26] This led to our postulate that the emergence of these mutations was due to exposure to sub-optimal levels of lamivudine. A few studies have also reported the occurrence of K65R in patients treated with a lamivudine/zidovudine-containing regimen, with an estimated prevalence of <0.5% from the Stanford University HIV Drug Resistance Database[23] and <6% from individual studies.[27–32]

K65R has a low genetic barrier and occurs through a point mutation involving a single purine–purine transition (AAA to AGA for non-subtype C viruses), which would explain its fast selection.[16] However, the frequency of K65R occurrence in the general population is usually low and this may be attributed to such factors as impaired viral replicative capacity of K65R mutants, reduced viral fitness in the presence of M184V, counter-selection with thymidine analogue-associated mutations (TAMs) and high potency of available regimens.[16,19,33,34] The high incidence observed in our study may be attributed to the continuous exposure of the virus to low doses of lamivudine ingested in breast milk. In a previous study, we showed that lamivudine and nevirapine were transferred from mothers to infants via breastfeeding and this led to the occurrence of acquired resistance to both drugs.[3,25] This was also reported in the PEPI-Malawi study, which showed that infants whose mothers were given post-partum PMTCT were likely to have multiclass drug resistance associated with the maternal regimen. In that study 11/37 infants developed resistance during breastfeeding, 7 of whom had the K65R mutation.[9] The mothers, however, were on a stavudine-containing regimen, which has been associated with selection of the K65R mutation. As reported in other studies,[9,35] we observed a reversion of the K65R mutation within 2–3 months of its emergence. This reversion could be a result of a reduction in the fitness of the K65R mutant, as reflected in its low replicative capacity, especially when it co-emerges with M184V or TAMs. The M184V mutation emerged in 5 (83%) of 6 infants with K65R and had not reverted by 9 months. An additional explanation could be the fact that these infants were weaned at 5.5 months and hence were no longer exposed to the low doses of lamivudine. This corresponded to the pharmacokinetic data, which showed plasma levels below the quantitative limit at 24 weeks. Four of the infants had initiated treatment at 6 months, but had not achieved viral suppression by 9 months. In addition, they had persisting reverse transcriptase inhibitor mutations, but with no re-emergence of the K65R mutation.

We further investigated factors associated with the K65R mutation. A low baseline CD4 cell count, early occurrence of any DRM and the presence of multiclass resistance were significantly associated with K65R emergence. Most of the infants were likely to have been infected prenatally, at delivery or 1 week post-partum. Since the majority of the infants with K65R mutations had first acquired mutations by 6 weeks of age, it is likely that cumulative accumulation of mutations could have been averted through immediate treatment initiation. In addition, the development of multiclass resistance in these infants further supports the need for early diagnosis and timely treatment of HIV-infected infants.[36]

In agreement with other studies was the absence of TAMs in almost all of the infants in whom K65R emerged.[14,16,19] TAMs are known to act antagonistically with K65R, restoring the catalytic activity of the reverse transcriptase enzyme initially lost due to the presence of this mutation, and for this reason they rarely co-emerge in the same genome.[16,19] In two instances, occurrence of TAMs followed reversion of K65R. We also observed the joint occurrence of NNRTI mutations K103N, G190A, K101E and Y181C with the K65R mutation. Previous studies have reported a synergistic fitness interaction between K65R and G190A/Y181C and a negative association with K103N.[16,37] This uncommon occurrence of K103N together with K65R in these infants may highlight the potential risk of unusual combinations of mutations as a result of exposure to sub-optimal doses of antiretroviral drugs in breast milk.

The findings of this study highlight the challenges in PMTCT strategies following paediatric HIV infection. The recent WHO guidelines recommending 'test and treat' strategies for HIV-infected infants are likely to reduce the occurrence of DRMs, including K65R. However, logistical and financial challenges in resource-limited settings leading to late HIV diagnosis may result in findings similar to those observed in this study. Currently, early diagnosis of HIV in infants is still a challenge in Kenya, and furthermore only a small percentage of HIV-infected infants (21%) are on therapy.[38] As a result there is need to strengthen early infant diagnosis programmes in Kenya to ensure timely access of HIV-1-infected infants to care and treatment.

There are limitations in this study. First, we were unable to conduct *in vitro* experiments to elucidate the molecular mechanism of K65R selection by lamivudine. Second, the follow-up period was short and insufficient to assess the long-term impact of the K65R mutation. Third, because of the small numbers we were not able to assess potential confounders of the predictors of K65R occurrence.

Finally, the use of conventional sequencing-based genotyping technique with sensitivity <20% may have limited the detection of minority K65R mutants and hence underestimated the prevalence of K65R in these infants. It is also possible that the K65R mutation could have persisted with time, but at low frequency.

In conclusion, a high incidence of K65R was observed among HIV-infected breastfeeding infants whose mothers were taking zidovudine, lamivudine and

either nevirapine or nelfinavir. We postulate that continuous exposure to low dosages of lamivudine through breast milk likely selected for this mutation. Future studies are, however, needed to elucidate the mechanism of K65R selection with sub-optimal doses of lamivudine. The emergence of the K65R mutation in these infants may have a negative impact on the infant's future regimen selection and treatment outcomes. The current recommendation for early diagnosis, immediate treatment and using a PI-based regimen is likely to prevent the emergence and accumulation of such mutations acquired through exposure to the maternal regimen.

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Transparency Declaration

None to declare.

Kenya Medical Research Institute and US CDC investigators were involved with all aspects of study design, data collection and analysis, interpretation of findings, report writing and the decision to submit the manuscript

Disclaimer

None to declare.

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Chapter 11

When prevention of mother-to-child HIV transmission fails: Preventing pre-treatment drug resistance in African children

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SUMMARY

The scale-up of antiretroviral prophylaxis to prevent mother-to-child transmission (PMTCT) of HIV has significantly reduced new pediatric infections in sub-Saharan Africa. However, among infants who become HIV-infected despite PMTCT, more than 50% have drug-resistant HIV. Given high levels of resistance, WHO recommends the use of protease inhibitors as part of first-line pediatric antiretroviral therapy (ART) to optimize treatment response, but costs and logistic challenges restrict access. A great concern is the current lack of ART options for children who experience virological failure. In this opinion paper, we argue that enhanced efforts are needed to help contain the emergence of pre-treatment resistance in children and hence improve ART outcomes. The vertical transmission of (drug-resistant) HIV can be prevented through enhancing ART adherence and frequent viral-load testing during pregnancy and throughout breast-feeding. Pre-treatment resistance, due to the use of sub-therapeutic infant prophylaxis or exposure to sub-optimal maternal ART through breast-feeding, can be prevented by the use of effective antiretroviral prophylaxis, based on either triple-drug combination or high-genetic barrier drugs, coupled with early infant diagnosis and prompt ART initiation. Further research is needed to assess programmatic barriers and cost-effectiveness of such strategies.

INTRODUCTION

In high- and some middle-income countries, effective programs to prevent mother-to-child transmission (PMTCT) using combination antiretroviral therapy (ART) for pregnant HIV-positive women and antiretroviral prophylaxis for HIV-exposed infants have resulted in 99-100% reduction in vertical transmissions [1]. However, in sub-Saharan Africa, the implementation of PMTCT programs has been less successful, with new pediatric infections having declined only by 21-86% between 2009-2015 [1]. Sub-Saharan Africa lags behind global trends because of several factors, including late or missed HIV testing, incident HIV infection in pregnant women, low uptake of or deferred treatment initiation, sub-optimal ART adherence pre- and post-partum, late identification and testing of HIV-exposed infants, amongst others [2–7].

The fact that children become HIV-infected despite the use of PMTCT ('failing PMTCT') is not only problematic because these infections are preventable, but also because infection during antiretroviral exposure increases the risk of acquiring drug-resistant HIV variants. Consequently, despite a decrease in the overall numbers of vertical HIV infections, the proportion of children who become infected and bear drug-resistant virus early in life is very high (35-64%) in the region [8–10]. The plight of these children is dire with high risk of failure on a suboptimal standard first-line ART regimen, absence of resistance tests to inform drug choices, and lack of alternative regimens in case of ART failure – all this in light of the need for lifelong effective therapy. In this opinion paper, we provide arguments that increased efforts are needed to prevent HIV drug-resistance early in life in the context of ongoing PMTCT programs and limited ART options for children.

Challenges in pediatric HIV management after failure of PMTCT

Studies in sub-Saharan Africa have reported that between 35-64% of HIV-infected infants have pre-treatment drug-resistant HIV (PDR), predominantly associated with the class of non-nucleoside reverse transcriptase inhibitors (NNRTIs) [8–10]. Although WHO has recommended protease inhibitor (PI)-based first-line ART since 2013, costs and logistics have largely hindered their accessibility. In 2015, about 77% of African children were still initiated on NNRTI-based regimens [11]. The recent development of a new lopinavir-ritonavir formulation as pellets (capsulated mini-tablets sprinkle formulation) may help improve access by overcoming cold-chain requirements for the liquid formulation and are easy to

administer with a simplified weight dosing, as opposed to the tablet formulations. On the other hand, limited production and high costs (~6x that of NNRTI-based regimens) still hinders access in these settings [12].

Even if access to PI-based regimens would be assured for young children, there are currently no good options available for children who experience ART failure; WHO recommends switching to either 2 NRTIs plus raltegravir or efavirenz (if age 3-10 years) or maintaining the child on the failing PI-based regimen [13]. These options are, however, suboptimal citing the high levels of both NNRTI and NRTI resistance in both pre-treated [8–10] and treated children [14].

Prevention of pre-treatment drug resistance in infants

Preventing PDR in infants will require strengthening the current PMTCT strategies as described below (Table 1).

Table 1: Summary of different strategies for optimization of PMTCT and prevention of PDR in HIV infected infants

Prevention of transmitted drug resistance from mothers to infants
• Enhanced adherence support and frequent viral-load monitoring during gestation and breast-feeding period to ensure sustained viral suppression and prevent acquired drug resistance in mothers
• Timely treatment switch upon virological failure to prevent acquired drug resistance
• Mothers initiating treatment late in pregnancy or those failing to achieve virological suppression by last trimester could be placed on INSTI-based regimens to facilitate fast viral-clearance
Prevention of PDR acquired either through exposures to sub-therapeutic infant prophylaxis or suboptimal maternal ART in breast-milk
• Use of triple ARVs prophylaxis instead of the currently recommended nevirapine mono or nevirapine/zidovudine dual therapy strategy
• Use of prophylactic drugs with high genetic barrier to resistance [a]
• Timely initiation of infant prophylaxis after birth to prevent drug resistance acquired from ingestion of suboptimal maternal drugs during breast-feeding
• Enhancing strategies for adherence of the prophylaxis regimens in infants and retention of mother-infant pairs

ARV, antiretroviral: ART, antiretroviral therapy; INSTI, integrase strand transfer inhibitior; PDR, pretreatment drug-resistant HIV

[a] This could include raltegravir which is currently being evaluated as an alternative PMTCT regimen in the IMPAACT P1110 study.

Strengthening the support framework for PMTCT to ensure better virological control during pregnancy and breast-feeding

Mothers with drug-resistant HIV can transmit the resistant strain to their infants during pregnancy, partus or breastfeeding [15]. Although there is limited data on the prevalence of HIV drug resistance in pregnant women it is likely that this might be increasing with wide-access to ART in this setting as observed in the general population [16]. For example, a recent study from Botswana showed that the rate of PDR in pregnant women had increased from <3% in 2012 to 9.7% in 2015 [17]. Moreover, about 29-50% of mothers have been reported to have detectable viremia during their last trimester [18,19], of whom 60-90% have drug-resistant HIV [20]. A recent study also showed that about 30% of women experience incident viremia during the post-partum phase highlighting an increased risk of transmission of drug-resistant variants during the extended period of breastfeeding [21], possibly attributable to sub-optimal adherence. A systematic review from 51 studies before the current era of lifelong triple ART for mothers in PMTCT programs also known as "option B+", reported that nearly 25% of women had sub-optimal adherence (<80%) during pre-partum with an even higher proportion of 47% during post-partum [22]. A complimentary review from 26 studies conducted between 2001-2012 reported that about 49% of mothers are lost-to-follow up during pregnancy and 33% of infants are lost-to-follow up within the first 12 weeks of life [23]. Studies report high attrition under option B+ in the early treatment phase and increased sub-optimal adherence during post-partum [2,6].

Therefore, to prevent vertical transmission of drug-resistant HIV, we propose to strengthen the PMTCT support framework in several ways. First, enhanced adherence and retention support can be achieved through peer support-based programs, such as the "expert mothers", enhanced adherence counseling for newly diagnosed mothers, male-partner involvement, phone calls/text-messages reminders and cash-based interventions [24–26]. However, wide-implementation is needed. Second, increased frequency of viral-load monitoring can help to ensure viral suppression during both pre-term and the breastfeeding period. The current guidelines recommend viral-load testing at 6 and 12 months post-partum [13], but this is not optimal for preventing the risk of HIV vertical transmission, citing high-levels of viral non-suppression at time of delivery [18,19] and during the breastfeeding period [20]. The minimal frequency of testing would preferably be every three months during partum and until cessation of breast-feeding, in line with the recommendations in high-income countries [27].

Further research is needed to assess the cost-effectiveness of these strategies.

Third, integrase-strand transfer inhibitors (INSTI) could be used in women who have high viremia in the last trimester and during breast-feeding, where possible and appropriate, to increase the chances for viral-suppression in order to maximize prevention of vertical transmission. The use of the recommended raltegravir-based regimen may be limited by high cost, although in the near future this could be replaced with low-cost dolutegravir as more data on its safety in pregnancy becomes available. Preliminary findings from the nationwide operational research in Botswana showed a safety profile comparable to efavirenz at conception, with long-term data expected in 2018 [28].

Overall, these efforts complement the recent WHO guidelines seeking to increase the likelihood of favorable pregnancy outcomes, through close monitoring of the mothers during the antenatal period [29].

Triple-drug combination antiretroviral prophylaxis in infants

Approximately 60-70% of HIV infections occur either *in utero* or intra-partum. However, due to late diagnosis, perinatally infected infants often receive sub-therapeutic regimens as prophylaxis, which increases the risk of selecting resistant strains [30]. Moreover, infants can also acquire resistance from ingestion of suboptimal doses of maternal regimens during breastfeeding [31]. Most of the current regimens given to mothers are known to enter breast-milk and are passed to their infants in suboptimal but therapeutically active doses [32].

The use of triple drug prophylaxis has a number of potential advantages. First, improved prevention of PDR, as the use of the currently recommended extended nevirapine mono- or nevirapine/zidovudine dual-prophylaxis brings the risk of selecting for NNRTI-resistance in infants [13]. This is due to the low genetic barrier to resistance of nevirapine, which requires only a single mutation for resistance to occur [33]. By contrast, the use of triple-drug prophylaxis, has been shown to effectively prevent resistance in early PMTCT studies [34]. Second, some studies suggest that triple-drug prophylaxis could be more efficacious for PMTCT than mono or dual-therapy [13,27,34], although data are conflicting. Indeed, based on expert opinion, most high-income countries now recommend the use of triple-drug prophylaxis (zidovudine/lamivudine/nevirapine) in high-risk infants born to women presenting late in care or with viremia by time of delivery [27]. WHO

already recommends this strategy as an alternative to the preferred zidovudine/nevirapine dual therapy when there is complexity in dosing of the latter [13]. In absence of good record keeping, coupled with lack of viral-load tests for pregnant and lactating mothers, it may not be feasible for programs to distinguish between low and high-risk infants. In our view, a standard triple-drug prophylaxis is probably a better standard strategy for PMTCT among HIV-exposed infants in these settings. Third, the use of triple-drug prophylaxis may serve as very early treatment to the infected, yet undiagnosed infants, which has the potential benefits of inducing long-term virological remission [35]. This may especially be advantageous in cases where birth testing is not feasible, and for the vertically infected infants who are missed by early infant diagnosis (EID) tests during the early window of infection [36]. Fourth, triple-drug prophylaxis of zidovudine/lamivudine/nevirapine is 40% cheaper than the standard WHO-recommended dual prophylaxis of zidovudine/nevirapine, and only 40% more expensive than nevirapine mono-prophylaxis [11,12]. Lastly, there are no major safety concerns with triple-drug prophylaxis of zidovudine/lamivudine/nevirapine relative to zidovudine/nevirapine dual-prophylaxis [37,38].

High genetic barrier antiretroviral prophylaxis in infants

The use of prophylactic drugs with a high genetic barrier to resistance, specifically protease inhibitors or INSTIs, provides an alternative strategy. However, there are safety concerns for use of PIs among pre-term infants and those <2 weeks of age [13]. Investigations are still ongoing for use of pediatric INSTI-based regimens as prophylaxis or treatment. Initial findings suggest a good safety profile for raltegravir in infants ≤6 weeks old [39]. Further studies are needed to assess the efficacy, safety and resistance of INSTI-based prophylaxis in infants.

Timely diagnosis of HIV infection and treatment initiation in infants

Prevention of PDR can also be enhanced by the timely identification of HIV-infected infants and prompt treatment initiation, especially in cases where it may not be feasible to implement the aforementioned infant prophylaxis strategies. The rate of early infant diagnosis and prompt treatment initiation is low in sub-Saharan Africa [7,40]. In 2015 only 51% of HIV-exposed infants were diagnosed within the first two months of life as per the previous WHO recommendations [7]. Potential remedies include the use of point-of-care testing, birth-testing, provider-initiated counseling and testing for all children and lactating mothers presenting in hospital for other ailments, use of text messages to speed relay of

EID results, family centered care and decentralization of pediatric ART services. However, more programmatic efforts are needed to ensure the effective implementation of these strategies [7,40].

In conclusion, high rates of PDR in infants who become HIV-infected despite the use of PMTCT are worrisome, especially considering the very limited ART options available for children in case of treatment failure. This could be prevented by strengthening the PMTCT framework, which not only prevents HIV transmissions but can also help prevent resistance in the infected infants. In our view, this could include: first, increasing the frequency of viral load monitoring in pregnant mothers to at least once every three months during pregnancy and breast-feeding; second, provision of integrase inhibitors to high-risk mothers with viremia in their last trimester or during breast-feeding; third, using triple-drugs or integrase inhibitors as infant prophylaxis; fourth, patient or programmatic tailor-made adherence and retention support; and fifth, timely identification of infected infants with prompt initiation of treatment. Operational research is urgently needed to assess cost-effectiveness and programmatic challenges of these strategies.

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Part III

Affordable diagnostics
for improved ART monitoring

Chapter 12

Stringent HIV viral load threshold for virological failure using dried blood spots: is the perfect the enemy of the good?

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Since 2013, World Health Organization recommends plasma HIV viral load (VL) as the preferred monitoring strategy of antiretroviral therapy (ART), complementing clinical and immunological criteria[1]. VL monitoring enables accurate detection of virological failure prompting timely adherence counseling and regimen switches before drug resistance accumulates[2], as well as prevention of inappropriate switching to more costly second-line regimens[2].

Despite significant progress [3], there are major obstacles to scaling up VL testing in low and middle-income countries (LMICs), including high costs, complex sample logistics, limited laboratory infrastructure and shortage of skilled staff. With point-of-care applications still largely unavailable [4], dried blood spots (DBS), rather than plasma, are propagated [5,6], because of the advantages of easy sample collection through finger-prick (eliminating phlebotomy and centrifugation) and easy shipment to a reference laboratory at ambient temperature (eliminating cold-chain transportation) [7-9]. However, potential limitations of DBS include lower amplification sensitivity (due to small sample volumes) and lower specificity (contribution by cell-associated viruses may overestimate VL) [7-10].

To account for these limitations, WHO previously recommended a VL threshold for virological failure of 3,000-5,000 cps/ml with DBS, compared to 1,000 cps/ml with plasma [1]. However, a 2014 WHO technical update revised the threshold with DBS to 1,000 cps/ml, citing provisional data from a review by Clinton Health Access Initiative on five commercial VL assays, which suggested sufficient sensitivities for this threshold [6].

We here assessed the public health implications of the revised, more stringent virological failure threshold with DBS, in terms of patient misclassification and clinical decision-making, using routine data from western Kenya. To this end, we projected the degree of patient misclassification, in terms of unnecessary test repeats and treatment switches, at the 1000 cps/ml, compared to the 5000 cps/ml threshold.

For this study, we used routinely collected standard plasma VL data from western Kenya collected real-time in the national web-based database (http://www.nascop.org/eid/viraloverall.php), obtained either using the Abbott real-time HIV-1 or Roche Cobas-Ampliprep/TaqMan (CAP/CTM) v2.0. We used reported sensitivity and specificity of five common VL test at the 1000 cps/ml threshold

from the CHAI review [6] and at the 5,000 cps/ml threshold from individual studies from sub-Saharan Africa, with the exception of Siemens kPCR (Table 1) [11-13]. We also included data for finger-prick based DBS which was available for the Abbott and NucliSENS assays[12,14]. All comparisons were done against plasma according to the assay standard procedures [11-14].

Under the current guidelines, the initial VL test (routine or targeted), if above threshold is followed by intensive adherence counseling and repeat test after 3 months. [1] Thus, a false-positive initial test will lead to an unnecessary repeat test, and a false-positive post-adherence test to an unnecessary regimen switch. A false-negative test will lead to undetected virological failure with potential for developing drug resistance. Projected misclassifications were calculated as follows; overall misclassification {(1-sensitivity) x (total positive) + (1-specificity) x total negatives)/total tests}, unnecessary repeats {(1-specificity) x (total negatives initial test)/total initial tests}, unnecessary switches {(1-specificity) x (total negatives post-adherence test)/total post-adherence tests} and undetected failures {(1-sensitivity) x (total positives)/total tests}.

The analysis included 79,566 VL test results from 78,604 patients receiving ART (97% on first-line) at 514 facilities in western Kenya, collected between January 2013 and November 2014. The reasons for testing were: routine initial test (n=56,006, 70.4%), targeted initial test (n=8517, 10.7%), post-adherence test (n=4,833, 6.1%), unrecorded (n=10,210, 12.8 %). VL test results were categorized as <1,000 cps/ml for 79.5%, 1,000-5,000 cps/ml for 4.6% and >5,000 cps/ml for 15.9%. Thus, at the 1,000 cps/ml threshold the proportion of patients with virological failure was 20.5%; among those, 22.4% had 1,000-5,000 cps/ml.

Table 1 summarizes the projected proportions of misclassifications for each VL assay at the 1,000 and 5,000 cps/ml thresholds. Overall, misclassifications were substantially more frequent at the 1,000 cps/ml (range 4.1% to 45.0%) threshold compared to 5,000 cps/ml (range 1.2 to 38.0%) and this varied between assays used.

The projected proportions of unnecessary repeat VL tests ranged from 1.8% (BioMérieux NucliSENS, Craponne, France) to 45.0% (Roche CAP/CTM-SPEX, Basel, Switzerland) at 1,000 cps/ml and 0.0% (BioMérieux NucliSENS) to 36.1% (Roche CAP/CTM-SPEX) at 5,000 cps/ml (Table 1). The 1,000 cps/ml threshold led to a

relative increase of 82.3% for Abbott real-time (Abbott, Abbott Park, IL) (venous blood); 56.9% for Abbott real-time (finger-prick); 96.4% for NucliSENS (venous blood); 100% for BioMérieux NucliSENS (finger-prick); and 19.8% for Roche CAP/CTM-SPEX, and a marginal decrease of 2.1% for Siemens Versant HIV-1 kPCR (Siemens, New York, NY).

The projected proportions of unnecessary switches ranged from 1.7% (BioMérieux NucliSENS) to 43.0% (Roche CAP/CTM-SPEX) at 1,000 cps/ml and 0.0% (BioMérieux NucliSENS) to 37.3% (Roche CAP/CTM-SPEX) at 5,000 cps/ml (**Table 1**). The 1,000 cps/ml threshold led to a relative increase of 81.8% for Abbott real-time (venous blood); 53.3% for Abbott real-time (finger-prick); 96.1% for BioMérieux NucliSENS (venous blood); 100% for BioMérieux NucliSENS (finger-prick); and 13.2% for Roche CAP/CTM-SPEX, and a decrease of 10.5% for Siemens Versant.

The projected proportions of undetected virological failure were low at either threshold ranging from 0.0% (Abbott real-time) to 3.9% (Roche CAP/CTM) (Table 1). The 1,000 cps/ml threshold led to a relative increase of 100% for Abbott real-time (venous blood), 42.9% for BioMérieux NucliSENS (venous blood) and 62.5% for Siemens Versant HIV-1 kPCR, and, by contrast, a decrease of 15.7% for Roche CAP/CTM-SPEX and 16.7% for BioMérieux NucliSENS (finger-prick).

Table 1: Expected clinical decisions based on viral load thresholds of virological failure from DBS at 1,000 and 5,000 cps/ml

Assay	VL threshold	N	Sensitivity	Specificity	Misclassification (%) based on expected decisions			
					Overall (%) n=79,566	Unnecessary repeats (%) n=54,006	Unnecessary switches (%) n=4,833	Undetected failures (%) n=79,566
[6, 13]Abbott Real-time HIV-1 (manual, m24sp and m2000sp) assays with m2000rt platform (venous blood)	<1000 cps/ml	1529	95.24	91.67	7.6	6.7	6.4	0.98
	<5000 cps/ml	149	100	98.6	1.2	1.2	1.2	0
[13]Abbott Real-time HIV-1 (manual, m24sp and m2000sp) assays with m2000rt platform (finger prick)	<1000 cps/ml	149	100	94.9	4.1	4.1	3.9	0
	<5000 cps/ml	149	100	97.8	1.9	1.8	1.8	0
[6]Biocentric: Generic HIV Charge Virale	<1000 cps/ml	531	94.86	55.16	36.7	35.9	34.4	1.1
	<5000 cps/ml					-	-	-
[6, 11]BioMérieux: NucliSENS EasyQ® HIV-1 v2.0 (venous blood)	<1000 cps/ml	1062	84.37	94.52	7.6	4.4	4.2	3.2
	<5000 cps/ml	610	88.5	99.8	2.0	0.16	0.17	1.8
[11]BioMérieux: NucliSENS EasyQ® HIV-1 v2.0 (finger prick)	<1000 cps/ml	612	88.7	97.8	4.1	1.8	1.7	2.3
	<5000 cps/ml	612	83	100	2.7	0	0	2.7
[6]Roche Molecular Systems: COBAS® AmpliPrep/COBAS® TaqMan® HIV-1 Test, v2.0 [free virus elution protocol]	<1000 cps/ml	229	81.02	96.74	6.5	2.6	2.5	3.9
	<5000 cps/ml	-				-	-	-
[6, 14]Roche Molecular Systems: COBAS® AmpliPrep/COBAS® TaqMan® HIV-1 Test, v2.0 (SPEX) protocol	<1000 cps/ml	2314	99.33	43.86	44.7	45.0	43.1	0.14
	<5000 cps/ml	839	99.0	55.0	38.0	36.1	37.4	0.16
[6, 12] HIV-1 RNA 1.0 Assay (kPCR)	<1000 cps/ml	144	90.97	87.76	11.8	9.8	9.4	2.0
	<5000 cps/ml	98	95.2	87.5	11.3	10.0	10.4	0.76

This study, based on routine VL data from western Kenya, demonstrates that DBS-based VL testing using the revised, stringent threshold for virological failure of 1,000 cps/ml (formerly 5,000 cps/ml), will substantially increase patient misclassification. This is projected to unnecessarily lead to 2-9 additional repeat VL tests and 2-6 additional regimen switches for every 100 VL tests performed, depending on the assay used, with only marginal or no reduction in undetected failures. This was with the exception of kPCR assay which had misclassification rates of ~10% which was unaltered at either thresholds. Our findings also indicate that VL assays that select for RNA either in extraction (e.g. Abbott-RealTime) or amplification (e.g. bioMérieux-NucliSENS), thus minimizing the contribution of cell-based viruses, are recommended for DBS, which is in line with a previous review [10].

Using estimates from the Abbott real-time system (preferred in-country DBS assay), the revised 1,000 cps/ml threshold would translate to 1,300-3,200 unnecessary repeat tests, at a total of 56,000 routine VL tests, within the study period. Extrapolating this to the national level, where an estimated 755,000 patients are on ART, an extra 16,600-41,000 repeat tests will be needed per year. The augmented demands in terms of adherence counseling, repeat VL tests and regimen switches will have important budget and resource implications. The additional laboratory costs and workload for adherence counselors and laboratory technicians would put further pressure on the already constrained resources for VL testing, potentially slowing down the scale-up [5]. Furthermore, increased unnecessary switches to second-line therapy, in the absence of confirmatory drug resistance testing, will augment drug costs and reduce future drug options. False-positive tests may also demotivate patients in their efforts for optimal drug adherence and impair the clinician's judgment of the credibility of laboratory results. Lastly, the more stringent VL threshold poses substantial challenges to developers of low-cost, in-house resistance assays for DBS to achieve optimal amplification sensitivity. This could result in reduced competition and longer-term dependence on relatively expensive VL options. In a recent pilot study from WHO/HIVResNet only 2 of 10 participating laboratories achieved an amplification sensitivity of 1,000 cps/ml [15].

Concerns have been raised that a higher VL threshold for virological failure could lead to potentially high numbers of patients with low VL levels that could be missed, thus continuing on a failing regimen [10]. Our real-life analysis, however,

indicates that this is unlikely to be the case, because of two reasons. First, in our study among patients with detectable viremia, the proportion of patients with low VL levels between 1,000 and 5,000 cps/ml was limited (4.6%), meaning that only few patients would potentially benefit from a more stringent threshold. Similar observations were noted in a Malawi study that evaluated the use of BioMérieux NucliSENS with DBS finger prick [14]. Second, the loss of sensitivity at a higher threshold of 5,000 cps/ml is only marginal for some of the assays used, i.e. BioMérieux NucliSENS, Roche CAP/CTM and Abbott real-time assays [11,12,14].

The study findings need to be interpreted with some caution. First, the comparative performance characteristics at the two thresholds were in some tests obtained from different studies. While variations in test-performance caused by site-related factors may lead to potential bias, this may be minimal as most of the studies used a relatively large sample size. Second, there was incomplete analysis for Roche CAP/CTM free-virus-elution protocol and Biocentric assays due to lack of comparative data at the 5,000 cps/ml threshold.

Finally, it needs to be recognized that a more stringent VL threshold could be advantageous in patients with low-level viremia, which are potentially at increased risk of residual immune dysregulation [17,18] and transmitting HIV [19]. Beside DBS, the use of other simplified specimen transportation methods such as dried plasma spots and dried tube specimens have also been exploited[8,16]. These alternatives however still require on-site centrifugation, which is usually lacking in most peripheral sites in LMICs. This in our view renders DBS the preferred technology in LMICs.

In conclusion, optimal efficiency will be critical to sustain effective HIV treatment in LIMCs. We here provided arguments that, from a public health perspective, a more stringent threshold of 1,000 cps/ml for DBS-based VL testing has potential disadvantages, in terms of an expected increase in unnecessary repeat testing and regimen switches. Whilst we strongly encourage further improvement of DBS-based technologies, at this stage of development, we recommend the use of a less stringent VF threshold, coupled with assays selecting for RNA in extraction or amplification. Don't let the perfect be the enemy of the good.

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Authors Contribution

TRW, SI, RH and CZ conceived and designed the study. SI performed the data analysis and drafted the manuscript. SI, RH and TRW provided valuable input to interpretation of the data and critically reviewed the paper for important intellectual content. All authors reviewed and approved the final version of the manuscript.

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Chapter 13

Field evaluation of a broadly sensitive HIV-1 in-house genotyping assay for use with both plasma and dried blood spot specimens in a resource-limited country

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ABSTRACT

HIV-1 drug resistance (HIVDR) assays are important tools in clinical management of HIV-infected patients on antiretroviral therapy (ART) and surveillance of drug-resistant variants at population levels. The high cost associated with commercial assays hinders their use in resource-limited settings. We adopted and validated a low-cost in-house assay using 68 matched plasma and dried blood spot (DBS) samples with a median viral load (VL) of 58,187 copies/ml, ranging from 253 to 3,264,850 against the commercial assay ViroSeq. Results indicated that the in-house assay not only had a higher plasma genotyping rate than did ViroSeq (94% versus 78%) but also was able to genotype 89.5% (51/57) of the matched DBS samples with VLs of ≥1,000 copies/ml. The sensitivity in detecting DR mutations by the in-house assay was 98.29% (95% confidence interval [CI], 97.86 to 98.72) on plasma and 96.54 (95% CI, 95.93 to 97.15) on DBS, and the specificity was 99.97% (95% CI, 99.91 to 100.00) for both sample types compared to ViroSeq. The minor DR mutation differences detected by the in-house assay against ViroSeq did not result in clinical significance. In addition, cost analysis showed that the in-house assay could reduce the genotyping cost by about 60% for both plasma and DBS compared to ViroSeq. This field condition evaluation highlights the potential utility of a cost-effective, subtype-independent, in-house genotyping assay using both plasma and DBS specimens for HIVDR clinical monitoring and population-based surveillance in resource-limited settings.

INTRODUCTION

There has been a tremendous increase in antiretroviral therapy (ART) in sub-Saharan Africa and other developing countries, largely due to the increased support by various multinational groups, such as the U.S. President's Emergency Plan for AIDS Relief (PEPFAR) and the Global Fund to Fight AIDS, Tuberculosis and Malaria (1–4). This has resulted in the significant reduction of HIV/AIDS-related morbidity and mortality among the 1.6 million patients currently on ART in the sub-Saharan region (1, 4). The long-term success of these ART programs, however, requires adequate monitoring of ART patients to ensure favorable treatment outcomes and minimize the development and transmission of HIV drug resistance (HIVDR), given the limited antiretroviral (ARV) drug regimen choices available in these settings (5–7). The cost and logistics involved in assays that are used to monitor HIVDR in ART patients remain challenging in resource-limited settings (5, 8, 9). Several initiatives are under way to establish alternative, less costly methods for laboratory-based ART patient monitoring, such as point-of-care CD4 testing, semiquantitative HIV viral load (VL) testing, and HIVDR testing using dried blood spots (DBS) (10–16). These parameters are routinely used in assessing treatment responses in resource-rich countries. HIVDR tests for patients on ART are not only important in monitoring individual patient treatment outcomes but also essential as public health tools in the routine assessment of the spread of drug-resistant variants at population levels (5, 17–19). Such data are vital in guiding a country's ARV drug regimen implementation strategy and in forecasting the need for new ARV drugs, especially in resource-limited settings where treatment options are limited (20–22).

Currently, there are two HIV-1 genotyping systems approved by the U.S. Food and Drug Administration (FDA), ViroSeq (Abbott Molecular, Abbott Park, IL) and TruGene (Siemens Healthcare Diagnostics, Deerfield, IL). These commercial systems are costly and were designed and approved to genotype HIV-1 subtype B viruses, which makes them less sensitive in genotyping HIV-1 variants in geographic areas where non-B subtypes and circulating recombinant forms (CRFs) are predominant (23, 24).

The preferred sample type used in these assays is either plasma or serum. This type of sample requires cold-chain conditions for collection, transportation, and storage (23) as well as separation of plasma from whole blood within 6 h

of blood collection. In addition, venous blood collection requires well-trained phlebotomists and poses a potential risk to health care workers for occupational HIV exposure from needle sticks. In resource-limited settings, inadequate health care infrastructure often necessitates the collection of blood samples from peripheral sites and transport to a reference facility where processing of samples and testing are carried out. These logistical issues make the conventional plasma specimen collection non-ideal for use in such settings. DBS offer an alternative specimen type to overcome these challenges: DBS do not require large amounts of blood and can be collected easily by finger or heel prick with minimal preprocessing procedures or risk to the health care worker. Moreover, they are easier to transport and store, do not require cold-chain transportation, and can be stored at ambient temperature for up to 2 weeks without compromising the genotyping efficiency (25, 26). Once air dried and properly packaged, they are considered noninfectious (27).

In order to mitigate the high cost associated with commercial genotyping tests and streamline the sample collection process, there have been systematic efforts to develop and evaluate in-house assays and adopt the assays for use with the DBS sample type, and several assays have shown promising results (24, 28–30). With the publication of the WHO manual for HIV drug resistance testing using dried blood spot specimens in 2010 and the promising results obtained from the in-house genotyping assays (31), we initiated a study to adopt and validate a broadly sensitive in-house assay (32, 33) against the FDA-approved ViroSeq HIV-1 drug resistance genotyping system using both plasma and DBS specimens.

MATERIALS AND METHODS

Study population

Plasma and DBS samples were obtained from HIV-1-positive mothers and children enrolled in the Kisumu Breastfeeding Study (KiBS), which assessed the efficacy of combined ART, mainly either nevirapine (NVP) with zidovudine (AZT) and lamivudine (3TC) or nelfinavir (NFV) with AZT and 3TC given from 34 weeks into the gestation period through 6 months postpartum for the prevention of mother-to-child transmission (PMTCT) (34). These included 39 samples from mothers collected at 6 months postpartum after at least 6 months on ART and 29 samples from infants exposed to maternal ART through breast milk. The VL

of these specimens ranged from 253 to 3,264,850 copies/ml. We also included seven DBS samples from a proficiency panel which were used to assess the reproducibility of DBS. These were replicates from a 14-member panel from the virological quality assurance program (VQA) at Chicago, IL, that had been contracted by the WHO-sponsored HIV proficiency testing program (30).

DBS and plasma collection and storage

Plasma was harvested through separation within 6 h from the time of whole-blood collection in EDTA-treated anticoagulant Vacutainer tubes (Becton, Dickinson, San Jose, CA) and stored at −60°C to −80°C. DBS were prepared by pipetting 50 µl of whole blood onto each of 5 spots on a 903 Whatman filter paper card (Schleicher & Schuell, Keene, NH). Filter papers were dried overnight in a clean biosafety cabinet and then placed individually in an air-impermeable zip-lock bag containing desiccants and a humidity indicator and stored at −30°C.

Nucleic acid extraction from plasma and dried blood spots

RNA from plasma was extracted using the FDA-approved ViroSeq HIV-1 genotyping system (Abbott Molecular, Abbott Park, IL) extraction protocol and the QIAamp viral RNA minikit (Qiagen Inc., Chatsworth, CA) for the in-house HIV-1 genotyping assay according to the manufacturer's instructions. Total nucleic acid (TNA) from DBS was extracted using a modified NucliSENS silica-based boom method (bioMérieux, Inc., Durham, NC) (29). Briefly, two 6-mm spots from each patient sample were cut and added into a tube containing 0.9 ml NucliSENS lysis buffer (bioMérieux, Inc., Durham, NC) and incubated at room temperature for 2 h under gentle rotation. After incubation, the tube was centrifuged for 2 min at 1,500 × g, and the supernatant was transferred into new 2-ml tubes. Nucleic acid was then extracted according to the manufacturer's instructions, eluted using 60 µl of elution buffer, and stored at −80°C until use.

HIV-1 drug resistance genotyping

HIV-1 genotyping was performed at the Kenya Medical Research Institute (KEMRI)/CDC HIV laboratory, which has been accredited as a National Drug Resistance Laboratory by the WHO HIV/ResNet group.

ViroSeq HIV-1 genotyping system

The FDA-approved ViroSeq assay amplifies an 1.8-kb fragment covering the entire protease region and codons 1 to 335 of the reverse transcriptase (RT) region.

This assay has an amplification detection sensitivity of 2,000 RNA copies/ml of plasma and a DR-associated mutation (DRM) detection sensitivity and specificity of 99.65% and 99.95%, respectively (35). Drug resistance genotyping was performed according to the manufacturer's instructions, and DR interpretations were conducted using both the ViroSeq HIV-1 genotyping system software, V2.6, and the Stanford genotyping resistance interpretation algorithm (http://sierra2.stanford.edu/sierra/servlet/JSierra) to allow for comparison with the in-house assay.

In-house genotyping assay

The in-house assay amplifies a 1,084-bp fragment of the HIV-1 pol gene encoding amino acids 6 to 99 of the protease region and codons 1 to 251 of the reverse transcriptase (RT) region. This assay was developed by the CDC, Atlanta, GA, for genotyping all HIV-1 group M subtypes and circulating recombinant forms (CRFs) and has been validated using samples collected from several PEPFAR-supported countries with multiple HIV-1 subtypes and CRFs (30, 32, 33). For this validation, we followed the procedure described by Yang et al. (32). Briefly, 15 μl of nucleic acid extracts from either plasma or DBS was subjected to a one-step RT-PCR using PRTM-F1, which is a 1:1 (wt/wt) combination of two forward primers (F1a, 5′-TGAARGAITGYACTGARAGRCAGGCTAAT, nucleotides [nt] 2057 to 2085 based on HXBII, and F1b, 5′-ACTGARAGRCAGGCTAATTTTTTAG, nt 2068 to 2092), and RT-R1 (reverse, 5′-ATCCCTGCATAAATCTGACTTGC, nt 3370 to 3348) (33; C. Yang, Z. Zhou, J. R. DeVos, and N. Wager, U.S. patent application 61/504,522) and the SuperScript III one-step RT-PCR system with Platinum Taq high-fidelity polymerase according to the manufacturer's protocol (Invitrogen, Carlsbad, CA). For the nested PCR, 4 μl of the RT-PCR product was then used with the inner primers PRT-F2 (forward, 5′-CTTTARCTTCCCTCARATCACTCT, nt 2243 to 2266) and RT-R2 (reverse, 5′-CTTCTGTATGTCATTGACAGTCC, nt 3326 to 3304) (32, 33) to yield an approximately 1.1-kb amplicon. Sequencing was then performed using six overlapping primers, and sequences were analyzed using the ABI 3100 genetic analyzer. Confirmation of base-calling and sequence editing were conducted using the Sequencher V4.5 (Genecodes) software. DR interpretation was performed using the Stanford genotyping drug resistance interpretation algorithm (v4.2.6) (http://sierra2.stanford.edu/sierra/servlet/JSierra) and using the International AIDS Society (IAS) 2011 mutation list (36) for confirmation.

Assay validation

Performance characteristics of this in-house assay were determined using the WHO/HIV ResNet guidelines for validation of in-house genotyping assays, which circumvent the lack of standard or reference methods for evaluating genotyping performance of DR assays (31). This included assessment of accuracy, precision, reproducibility, and amplification sensitivity as minimal requirements; linearity and sensitivity, though not assessed in this validation, are also considered.

Accuracy: To assess accuracy, 53 plasma and 52 DBS sample sequences obtained from the in-house assay were compared to the plasma sample sequences obtained from ViroSeq. Accuracy was determined by the degree of concordance between the 66 DRMs identified by ViroSeq and the in-house assay based on the IAS 2011 mutation list (36).

Sensitivity: Sensitivity was assessed using 68 samples with VL ranges from 253 to 3,264,850 copies/ml, and the genotyping sensitivity is defined as the VL copy ranges at which ≥95% of the samples were successfully genotyped.

Precision and reproducibility: Precision was assessed using 10 samples with 3 to 5 replicates (5 samples with 5 replicates and 5 samples with 3 replicates), and the reproducibility was determined using 12 samples with at least 2 replicates (5 samples with 5 replicates and 7 duplicate samples). In addition, seven DBS replicates from a 14-member proficiency testing panel from VQA at Chicago, IL, were used to assess the reproducibility for DBS specimens. The replicates in the panel had been shipped under two different temperature conditions. The reproducibility test was performed by two technicians and in some cases utilizing different kit lots. Both precision and reproducibility were determined by the degree of concordance of DR-associated mutations as well as the degree of nucleotide sequence identity.

Contribution of proviral DNA from DBS: The frequency of amplification of proviral DNA from DBS was investigated using the in-house assay on eight samples with VLs ranging from 15,508 to 3,264,850 copies/ml. These 8 samples were run in the presence and absence of the RT-PCR murine leukemia virus (MuLV) enzyme.

Phylogenetic analysis

Phylogenetic analysis was performed using the neighbor-joining method in MEGA4 software (37). The evolutionary distances were computed using the maximum composite likelihood method in units of the number of base substitutions per site. The tree was then generated by the neighbor-joining

method from a nucleotide alignment of 751 positions devoid of gaps, and tree topology was confirmed by bootstrapping analysis using 1,000 replicates. Pairwise alignment to assess nucleotide sequence identity between matched plasma and DBS and those plasma sequences obtained from the ViroSeq assay was performed using the EMBOSS pairwise alignment tool (http://www.ebi.ac.uk/Tools/psa/emboss_needle/) in the needle global method for whole-length alignment (with default gap-penalty values).

Statistical analysis

The sensitivity and specificity of the in-house assay for both plasma and DBS were assessed against the ViroSeq system on 66 DRMs as identified in the IAS 2011 mutation list (36). Quantitative variables are expressed as means ± standard deviations (SDs) unless otherwise stated. The McNemar test was then used to assess for significance in the discordant mutations between the in-house and the ViroSeq assays for both sample types. Precision and reproducibility were assessed using the Cohen kappa statistic. Agreement was interpreted as weak ($0.400 > \kappa \geq 0.200$), moderate ($0.600 > \kappa \geq 0.400$), strong ($0.800 > \kappa \geq 0.600$), nearly perfect ($1.00 > \kappa \geq 0.800$), and perfect ($\kappa = 1.000$). Sample size was estimated using Buderer's formula (38) with the following assumptions: HIVDR prevalence of 9.3% based on a previous study in Kenya (39), anticipated sensitivity equivalent to that obtained with the ViroSeq system of 99.65 (35), and a two-sided test with an alpha value of 0.05 and a precision value of 0.05. A minimum sample size of 60 was then required in order to obtain a sensitivity equivalent to that of the ViroSeq system.

Quality control and assurance

The quality of the test runs was ensured by the inclusion of positive and negative controls in each run. Sequence quality was confirmed by using the WHO sequence quality assessment tool (SQUAT) (40) to screen ambiguous nucleotides, frameshifts, insertions, and deletions of the sequences. In addition, ambiguous and atypical amino acids, stop codons, and frameshifts were also screened at the amino acid level. SQUAT was also used to check for cross-contamination by calculating pairwise genetic distances which were further crosschecked by the PAUP tool (41). The KEMRI/CDC HIV research laboratory, where the analysis was performed, participates twice a year in external quality assurance (EQA) programs offered by VQA and the College of American Pathologists (CAP) for HIV plasma genotyping for both the in-house and ViroSeq assays. At the time that

this study was conducted, the performances of all the proficiency testing panels were satisfactory. The laboratory is also accredited with the ISO 15189 standard and by WHO ResNet for HIVDR genotyping.

Ethical considerations

Ethical review committees of the Kenya Medical Research Institute (KEMRI) and the Institutional Review Board of the U.S. Centers for Disease Control and Prevention (CDC), Atlanta, GA, approved this study. All mothers in the KiBS study provided written informed consent that included parental consent for their infants.

Nucleotide sequence accession numbers: Sequences from this study were submitted to GenBank, and their accession numbers are JQ914045 to JQ914103.

RESULTS

Viral load determination

Among the 68 plasma samples, the mean plasma VL was 330,855 copies/ml with a median of 58,187 copies/ml, ranging from 253 to 3,264,850 copies/ml. Of these, 44 had VLs of >10,000 copies/ml (median, 374,074), 13 ranged from 1,000 to 10,000 copies/ml (median, 4,040), and the remaining 11 had <1,000 copies/ml (median, 632).

Plasma genotyping sensitivity

Of the 68 plasma samples used to assess genotyping sensitivity, 100% (95% confidence interval [CI], 93.5 to 100) of all the 44 samples with VLs of >10,000 were genotyped by both assays. All the 13 samples with VLs between 1,000 and 10,000 copies/ml were genotyped with the in-house assay (100%; 95% CI, 80.7 to 100), and 8 (61.5%; 95% CI, 36.1 to 83.1) were genotyped with the ViroSeq assay. For those 11 samples with VLs of <1,000 copies/ml, the in-house assay was able to genotype 7 (63.6%; 95% CI, 36.2 to 85.9) while 1 (9.1%) was genotyped by the ViroSeq system (Table 1).

DBS genotyping sensitivity

After establishing better sensitivity for plasma samples with lower VL measurements in the in-house assay than in the ViroSeq assay, the in-house

assay was then used to assess genotyping sensitivity using the matched 44 DBS samples. All the 44 DBS samples (100%; 95% CI, 93.5 to 100) with VLs of >10,000 copies/ml were genotyped by the in-house assay. In addition, 7 (53.8%; 95% CI, 29.1 to 77.3) of the 13 DBS samples with VLs between 1,000 and 10,000 copies/ml were also genotyped, while only 1 of the 11 DBS samples with VLs of <1,000 copies/ml was genotyped (Table 1).

Table 1: Patient characteristics and genotyping efficiency from Plasma and DBS using the In-house Assay comparing with the ViroSeq commercial assay

ID[a]	Plasma viral load (copies/ml)	ART (regimen)[b]	Length of DBS storage (mo)	Plasma genotype (viroseq)	Plasma genotype (in-house)	DBS genotype (in-house)	HIV-1 Subtype
1-0472-8v7	3,264,850	AZT+ABC+KAL	0-12	+	+	+	A
1-0079-v8	1,408,848	3TC+AZT+NVP	37-48	+	+	+	A
1-0119-4v11	1,220,862	-	25-36	+	+	+	AD
1-0230-2v7	1,133,811	3TC+AZT+NVP	25-36	+	+	+	CRF10_CD
1-0119-4v7	1,023,877	-	25-36	+	+	+	AD
1-0357-6v11	961,300	AZT+ABC+KAL	0-12	+	+	+	A
1-0457-9v5	834,528	-	13-24	+	+	+	A
1-0410-4V9	821,222	AZT+ABC+KAL	0-12	+	+	+	AD
1-0437-5v7	810,648	-	0-12	+	+	+	A
1-0357-6v8	718,896	3TC+AZT+NVP	13-24	+	+	+	A
0-0140-4v17	714,157	3TC+AZT+NVP	25-36	+	+	+	A
1-0085-1v8	708,158	3TC+AZT+NVP	37-48	+	+	+	C
1-0496-6v7	619,222	-	0-12	+	+	+	A
1-0360-1v7	587,486	3TC+AZT+NFV	13-24	+	+	+	A
1-0053-3v11	562,910	3TC+AZT+NVP	25-36	+	+	+	A
1-0105-8v12	551,800	3TC+AZT+NVP	13-24	+	+	+	A
1-0437-5v11	530,000	-	0-12	+	+	+	A
1-0066-8v9	467,363	3TC+AZT+NVP	25-36	+	+	+	A
1-0144-5v12	431,147	3TC+AZT+NVP	13-24	+	+	+	D
0-0106-9v16	418,017	3TC+AZT+NVP	25-36	+	+	+	A
0-0137-6v16	402,754	3TC+AZT+NVP	37-48	+	+	+	CRF10_CD
0-0056-6v16	380,117	3TC+AZT+NVP	37-48	+	+	+	C
1-0230-2v5	368,032	-	37-48	+	+	+	CRF10_CD
0-0074-8v16	323,445	3TC+AZT+NVP	37-48	+	+	+	A
1-0410-4v7	320,945	-	13-24	+	+	+	AD
1-0496-6v3	303,144	-	13-24	+	+	+	A
0-0158-1v16	292,850	3TC+AZT+NVP	37-48	+	+	+	A
0-0113-8v17	290,549	3TC+AZT+NVP	25-36	+	+	+	A
1-0317-8v8	192,450	-	13-24	+	+	+	CRF10_CD
0-0150-3v16	117,493	3TC+AZT+NVP	37-48	+	+	+	A
0-0230-2v20	111,021	3TC+AZT+NVP	13-24	+	+	+	CRF10_CD

0-0472-8v3	106,234	3TC+AZT+NFV	13-24	+	+	+	A
1-0289-1v5	87,142	-	25-36	+	+	+	A
0-0200-6v17	72,112	3TC+AZT+NVP	25-36	+	+	+	A
0-0266-4v16	44,262	3TC+AZT+NFV	25-36	+	+	+	A
0-0496-6v16	31,511	3TC+AZT+NFV	0-12	+	+	+	A
0-0113-8v16	30,889	3TC+AZT+NVP	37-48	+	+	+	A
1-0066-8v1	26,412	-	37-48	+	+	+	A
0-0200-6v16	25,395	3TC+AZT+NVP	25-36	+	+	+	A
0-0127-4v16	21,431	3TC+AZT+NVP	37-48	+	+	+	D
1-0317-8v12	21,000	3TC+AZT+NFV	0-12	+	+	+	CRF10_CD
1-0066-8v7	17,591	3TC+AZT+NVP	37-48	+	+	+	A
0-0182-1v16	15,508	3TC+AZT+NVP	37-48	+	+	+	A
0-0472-8v17	11,381	3TC+AZT+NFV	0-12	+	+	+	A
1-0079-3v5	7,398	-	37-48	+	+	+	A
0-0472-8v16	6,923	3TC+AZT+NFV	0-12	+	+	+	A
0-0157-0v16	6,084	3TC+AZT+NVP	37-48	+	+	+	A
0-0073-7v16	5,547	3TC+AZT+NVP	37-48	+	+	+	A
0-0120-7v16	5,434	3TC+AZT+NVP	37-48	+	+	-	D
1-0517-4V5	5,148	-	13-24	+	+	+	A
1-0360-1v12	4,040	AZT+ABC+KAL	0-12	+	+	+	A
0-0036-2v16	1,365	3TC+AZT+NVP	37-48	-	+	-	A
0-0264-2v16	1,313	3TC+AZT+NVP	25-36	-	+	-	A
0-0020-4v16	1,106	3TC+AZT+NVP	37-48	+	+	+	A
0-0517-4v16	1,026	3TC+AZT+NFV	0-12	-	+	-	A
0-0519-6v16	1,024	3TC+AZT+NFV	0-12	-	+	-	G
0-0100-3v16	1,006	3TC+AZT+NVP	37-48	-	+	-	A
0-0475-1v16	961	3TC+AZT+NFV	0-12	-	+	-	A
0-0433-1v16	891	3TC+AZT+NFV	13-24	+	+	+	A
0-0212-0v16	792	3TC+AZT+NVP	37-48	-	+	-	A
0-0093-1v16	737	3TC+AZT+NVP	37-48	-	+	-	A
0-0042-0v16	666	3TC+AZT+NVP	37-48	-	+	-	A
0-0523-2v17	632	3TC+AZT+NVP	0-12	-	+	-	D
0-0227-7v16	475	3TC+AZT+NVP	37-48	-	+	-	A
0-0291-5v16	373	3TC+AZT+NFV	13-24	-	-	-	-
0-0276-6v16	352	3TC+AZT+NFV	13-24	-	-	-	-
0-0024-8v16	260	3TC+AZT+NVP	37-48	-	-	-	-
0-0500-5v16	253	3TC+AZT+NFV	0-12	-	-	-	-

[a]Study sample identifiers (IDs) with specific visit codes (shown by "v"). Patient identifiers starting with 0 are samples collected from the mothers, while those starting with 1 are samples collected from infants. Some of the samples included in the study were collected from the same participants at different study visit points.
[b]Abbreviations: TN, treatment naïve; 3TC, lamivudine; AZT, zidovudine; NVP, nevirapine; KAL, lopinavir-ritonavir (Kaletra); ABC, abacavir; NFV, nelfinavir.

Concordance of the two assays in detecting drug resistance-associated mutations in plasma samples

The accuracy in detecting DRMs in plasma was determined by using the 53 sequences generated by the ViroSeq system. Two hundred thirty-four DRMs were observed in these 53 sequences, including 68 DRMs in the RT gene and one major and 165 minor mutations in the protease gene. Of these DRMs, 230 were detected in the sequences generated by the in-house assay, which yields an analytic accuracy of 98.29% (95% CI, 97.86 to 98.72) (Tables 2 and 3). Of the four discordant DRMs between the two assays, three were due to mixtures in the RT gene. M184IV and M184MV were detected as mixtures in the ViroSeq system but were nonmixture mutations in the in-house assay (M184V). In contrast, G190AG was detected as a mixture in the in-house assay, but it was a nonmixture in the ViroSeq system (G190A). The one remaining discordant mutation was a minor mutation (G16E) in the protease gene, which was missed by the in-house assay. None of these discordant DRMs were of clinical significance when the sequences were analyzed with the HIValg program using the HIVdb v6.2.0 program deployed at the Stanford HIV Drug Resistance Database. Pairwise comparison of nucleotide substitutions at the DRM sites also indicated high concordance, with only five (0.7%) nonsynonymous substitutions. Overall, compared to the ViroSeq system, the in-house assay gave high specificity in detection of DRMs (99.97%; 95% CI, 99.91 to 100.03) as well as excellent positive and negative predictive values (99.57%; 95% CI, 99.35 to 99.78; 99.88%; 95% CI, 99.76 to 99.99, respectively) (Table 3). Statistical analyses using the McNemar test on paired results for DRMs also supported the excellent performance of the in-house assay compared to the ViroSeq system ($\chi 2 = 1.80$, $P = 0.375$). The excellent performance by the in-house assay was further supported by stratification analysis of DRMs for sensitivity and specificity (sensitivity $\chi 2 = 4$, $P = 0.125$, and specificity $\chi 2 = 1.0$, $P = 1.0$). At the nucleotide level, the mean nucleotide sequence identity was 99.5% for the in-house assay compared to the ViroSeq system. The minor differences observed in sequence identity were mainly caused by mixture bases. We identified 126 mixture base differences among the 53 sequences analyzed; of these, 93 (74%) were detected by the in-house assay and 86 (70%) were detected by the ViroSeq system.

Concordance of the two assays in detecting drug resistance mutations in DBS samples

Based on the satisfactory results obtained with the in-house assay in comparison with the ViroSeq system as well as the higher sensitivity in genotyping plasma samples with lower VLs with the in-house assay, we next genotyped and analyzed the 52 matched DBS samples using the in-house assay only and compared the genotyping results with those obtained from plasma samples by the ViroSeq system. Compared to sequences obtained from plasma samples using the ViroSeq system, the in-house assay gave a sensitivity of 96.54% (95% CI, 95.93 to 97.15) in DRM detection (Tables 2 and 3). A total of 231 mutations were observed in the ViroSeq system. Of these, 223 were identified in DBS sequences by the in-house assay. Among the eight discordant DRMs, five occurred in the RT gene, four of which were caused by mixtures (Y181CY, K103KN, T215FIST, and MI84IMV) while the remaining one (K70KR) was not detected in the DBS sample by the in-house assay. The remaining three discordant mutations were detected in the protease gene, two of which occurred as mixtures at minor DR positions (K20KR and M36IV) while the other one was absent (G16E) in the in-house assay. In addition, one extra DRM (Y181CY) was present only in one DBS sample. Despite the DRM detection differences, they were not clinically significant even for the patient with the Y181CY mutation since this patient had already had a K013KN mutation, which led to high-level resistance to nonnucleoside reverse transcriptase inhibitors (NNRTI). Pairwise comparison of nucleotide substitution at DRM sites was also highly concordant for DBS with only 12 (1.7%) nonsynonymous substitutions. Similar to plasma, the in-house assay also gave near-perfect specificity in detecting DRMs in DBS samples (99.97%; 95% CI, 99.91 to 100.03) with excellent positive (99.55; 95% CI, 99.33 to 99.78) and negative (99.75; 95% CI, 99.58 to 99.92) predictive values (Table 3). McNemar test analysis on paired results for DRM detection confirmed the excellent concordant results ($\chi 2 = 5.44$, $P = 0.04$) and great sensitivity and specificity ($\chi 2 = 8$, $P = 0.008$, and $\chi 2 = 1.0$, $P = 1.0$), respectively. As expected, at the nucleotide level, the in-house assay also gave excellent mean nucleotide identity compared to the plasma ViroSeq system (99.5%). The minor differences from the McNemar test as well as nucleotide sequence identity were caused by mixture bases, of which the in-house assay using DBS was able to detect 79 of 126 (63%), compared to 70% for the ViroSeq system.

Table 2: Comparison of drug resistance mutations identified by the in-house assay from plasma and DBS specimens with those identified by ViroSeq from plasma specimens

	Protease gene			Reverse Transcriptase gene		
ID	**Viroseq Plasma**	**In-house Plasma**	**In-house DBS**	**Viroseq Plasma**	**In-house Plasma**	**In-house DBS**
0-0020-4v16	K20R,M36I,H69K	K20R,M36I,H69K	K20R,M36I,H69K			
0.0056.6v16	M36I,H69K	M36I,H69K	M36I,H69K	**K103N**	**K103N**	**K103N**
0-0073-7v16	G16E,M36I,H69K	G16E,M36I,H69K	G16E,M36I,H69K	**V108I,Y181L,M184V**	**V108I,Y181L,M184V**	**V108I,Y181L,M184V**
0-0074-8v16	K20R,M36I,H69K	K20R,M36I,H69K	K20R,M36I,H69K	**G190A**	**G190A**	**G190A**
0-0106-9v16	M36I,H69K	M36I,H69K	M36I,H69K	**M184V**	**M184V**	**M184V**
0-0113-8v16	M36I,L63P,H69K	M36I,L63P,H69K	M36I,L63P,H69K	**K103N,M184V**	**K103N,M184V**	**K103N,M184V**
0-0113-8v17	M36I,L63P,H69K	M36I,L63P,H69K	M36I,L63P,H69K			
0-0120-7v16	M36I,L63P,V77I	M36I,L63P,V77I	_			_
0-0127-4v16	K20R,M36I,L63P,H69K	K20R,M36I,L63P,H69K	K20R,M36I,L63P,H69K			
0-0137-6v16	D60E,I64V,V77I	D60E,I64V,V77I	D60E,I64V,V77I			
0-0140-4v17	M36I,**M46L**,L63LP, H69K	M36I,**M46L**,L63LP, H69K	M36I,**M46L**,L63LP, H69K			
0-0150-3v16	M36I,H69K	M36I,H69K	M36I,H69K			
0-0157-0v16	M36I,K20R,H69K	M36I,K20R,H69K	M36I,K20R,H69K			
0-0158-1v16	M36I,H69K	M36I,H69K	M36I,H69K	**K103KN**	**K103KN**	**K103KN**
0-0182-1v16	K20R,M36I,H69K	K20R,M36I,H69K	K20R,M36I,H69K	**M184V,Y188L**	**M184V,Y188L**	**M184V,Y188L**
0.0200.6V16	K20R,M36I,H69K	K20R,M36I,H69K	K20R,M36I,H69K	**Y181C,M184V**	**Y181C,M184V**	**Y181CY,M184IMV**
0-0200-6v17	K20R,M36I,H69K	K20R,M36I,H69K	K20R,M36I,H69K			
0-0230-2v20	K20R, M36I,L63P, I64V	K20R, M36I,L63P, I64V	K20R, M36I,L63P, I64V	**K103N**	**K103N**	**K103N**
0-0266-4v16	G16E,M36I,H69K	G16E,M36I,H69K	G16E,M36I,H69K			
0-0433-1v16	M36I,H69K	M36I,H69K	M36I,H69K	**M184V**	**M184V**	**M184V**
0-0472-8v3	G16E ,K20KR,M36I,H69K	G16E,K20KR,M36I,H69K	G16E,K20R,M36I,H69K			
0-0472-8v16	G16E,K20R,M36I,H69K	G16E,K20R,M36I,H69K	G16E,K20R,M36I,H69K	**K103N,M184MV**	**K103N,M184V**	**K103N,Y181CY,M184MV**
0-0472-8v17	G16E,K20R,M36I,H69K	G16E,K20R,M36I,H69K	G16E,K20R,M36I,H69K	**K103N,M184V**	**K103N,M184V**	**K103KN,M184MV**
0-0496-6v16	M36I,H69K	M36I,H69K	M36I,H69K			
1-0053-3v11	M36I,H69K	M36I,H69K	M36I,H69K			
1-0066-8v1	K20R,M36I,D60E,I62V,H69K	K20R,M36I,D60E,I62V,H69K	K20R,M36I,D60E,I62V,H69K			
1-0066-8v7	K20R,M36I,D60E,I62V,H69K	K20R,M36I,D60E,I62V,H69K	K20R,M36I,D60E,I62V,H69K	**D67DN,G190A,T215F**	**D67DN,G190A,T215F**	**D67DN,G190A,T215F**
1-0066-8v9	K20R,M36I,D60E,I62V,H69K	K20R,M36I,D60E,I62V,H69K	K20R,M36I,D60E,I62V,H69K	**D67DN,K70KR,K101KQ, K103KN,M184V,G190AG, T215FS,K219EK**	**D67DN,K70KR,K101KQ, K103KN,M184V,G190AG ,T215FS,K219EK**	**D67DN,K70KR,K101KQ, K103KN,M184V,G190AG, T215FIST,K219EK**

1-0079-3v5	G16E,M36I,H69K	G16E,M36I,H69K	G16E,M36I,H69K	**K65KR,K101EK,Y181CY, M184MV,G190A**	**K65KR,K101EK,Y181CY, M184MV,G190A**	**K65KR, K101EK, Y181CY, M184IMV, G190A**
1-0079- v8	G16E,M36I,H69K	G16E,M36I,H69K	G16E,M36I,H69K	**K101E,M184V,G190A**	**K101E,M184V,G190A**	**K101E,M184V,G190A**
1-0085-1v8	M36I,H69K,I93L	M36I,H69K,I93L	M36I,H69K,I93L			
1-0105-8v12	M36I,D60E,I62V,H69K	M36I,D60E,I62V,H69K	M36I,D60E,I62V,H69K			
1-0119-4v7	M36I, H69K	M36I, H69K	M36I, H69K			
1-0119-4v11	M36I, H69K	M36I, H69K	M36I, H69K			
1-0144-5v12	M36I,I64V	M36I,I64V	M36I,I64V			
1-0230-2v7	K20R, M36I,R41K, L63P, I64V	K20R, M36I,R41K, L63P, I64V	K20R, M36I,R41K, L63P, I64V	**M184V**	**M184V**	**M184V**
1-0230-2v7	K20R, M36I,L63P, I64VF	K20R, M36I,L63P, I64V	K20R, M36I,L63P, I64V	**M184V**	**M184V**	**M184V**
1-0289-1v5	M36I,H69K	M36I,H69K	M36I,H69K	**M184V**	**M184V**	**M184V**
1-0317-8v8	M36I,I62V,I64V	M36I,I62V,I64V	M36I,I62V,I64V	**Y181C, M184V**	**Y181C, M184V**	**Y181C,M184V**
1-0317-8v12	M36I,I62V,I64V	M36I,I62V,I64V	M36IV,I62V,I64V	**K70KR,Y181C, M184V**	**K70KR,Y181C, M184V**	**Y181C, M184V**
1-0357-6v8	M36I, H69K	M36I, H69K	M36I, H69K	**M184V,G190A**	**M184V,G190AG**	**M184V,G190A**
1-0357-6v11	M36I,D60E, H69K	M36I,D60E, H69K	M36I,D60E, H69K	**A98AG,K103S, M184V, G190A,T215Y**	**A98AG,K103S, M184V, G190A,T215Y**	**A98AG,K103S, M184V, G190A,T215Y**
1-0360-1v7	G16E,K20R,M36I,I62V,H69K	G16E,K20R,M36I,I62V,H69K	G16E,K20R,M36I,I62V,H69K	**M184V**	**M184V**	**M184V**
1-0360-1V12	G16E,K20R,M36I,I62V,H69K	G16E,K20R,M36I,I62V,H69K	G16E,K20R,M36I,I62V,H69K		**M184MV**	
1-0410-4v7	M36I,L63P,H69K	M36I,L63P,H69K	M36I,L63P,H69K	**M184MV,T215Y**	**M184MV,T215Y**	**M184MV,T215Y**
1-0410-4V9	M361,L63P,H69K	M361,L63P,H69K	M361,L63P,H69K	**T215D**	**T215D**	**T215D**
1-0437-5v7	M36I,L63P,H69K	M36I,L63P,H69K	M36I,L63P,H69K	**M184IV**	**M184V**	**M184V**
1-0437-5v11	M36I,L63P,H69K	M36I,L63P,H69K	M36I,L63P,H69K			
1-0457-9v5	K20R,M36I,H69K	K20R,M36I,H69K	K20R,M36I,H69K	**K65KR,M184MV**	**K65KR,M184MV**	**K65KR,M184MV**
1-0472-8V7	G16E,K20R,H69K	G16E,K20R, H69K	G16E,K20R, H69K	**K65KR,K103N, Y181CY,M184MV**	**K65KR,K103N, Y181CY,M184MV**	**K65KR,K103N, Y181CY,M184V**
1-0496-6v3	G16E,K20R,M36I,H69K	K20R,M36I,H69K	K20R,M36I,H69K	**K65KR,M184MV**	**K65KR,M184MV**	**K65KR,M184MV**
1-0496-6v7	M36I,H69K	M36I,69K	M36I,H69K	**M184V**	**M184V**	**M184V**
1-0517-4V5	M36I,H69K	M36I,H69K	M36I,H69K	**M184V**	**M184V**	**M184V**

[a]Major drug resistance mutations against protease and reverse transcriptase inhibitors are shown in bold, and discordant drug resistance mutations are underlined.
[b]ID, identifier.

Table 3: Performance characteristics of the in-house genotyping assay compared to the Viroseq system in detection of drug resistance mutations

In-house (*n*)	No. of DRMs by Viroseq result		% (95% CI[a])				Mean nucleotide score (%)
	Positive	Negative	Sensitivity %	Specificity %	PPV%	NPV%	
Plasma + n=53	230	1	98.29 (97.86-98.72)	99.97 (99.91-100.03)	99.57 (99.35-99.78)	99.88 (99.76-99.99)	99.5%
-	1	3263					
DBS + n=52	223	1	96.54 (95.93-97.15)	99.97 (99.91-100.03)	99.55 (99.33-99.78)	99.75 (99.58-99.92)	99.5%
-	8	3200					

[a] CI, confidence interval; PPV, positive predictive value; NPV, negative predictive value

Table 4: Precision and reproducibility of genotyping using plasma and DBS with the in-house assay

	No of samples	No of replicates	κ value	95% CI	[a]Interpretation	Mean nucleotide identity score ±SD
Precision						
Plasma	10	40	0.974	0.960-0.989	NP	99.4 ±0.33
DBS	10	40	0.967	0.960-0.968	NP	99.2±0.59
Reproducibility						
Plasma	10	39	0.975	0.956-0.980	NP	99.3±0.35
DBS	10	53	0.992	0.991-0.995	NP	99.1±0.59

[a] Interpretation for kappa statistic NP= near perfect

Precision and reproducibility

Using the WHO criteria for DBS genotyping method validation, the in-house assay demonstrated a high precision and reproducibility in both DBS and plasma specimen types (Table 4). Overall agreement for precision was nearly perfect

with a kappa score of 0.974 (95% CI, 0.960 to 0.989) for plasma and 0.967 (95% CI, 0.960 to 0.968) for DBS. The mean nucleotide sequence identity score for precision was 99.4% ± 0.33% in plasma and 99.2% ± 0.59% in DBS. Similar results were also obtained in the reproducibility assessment, where the kappa score was nearly perfect: 0.975 (95% CI, 0.956 to 0.980) for plasma and 0.992 (95% CI, 0.991 to 0.995) for DBS. The mean nucleotide sequence identity was 99.3% ± 0.35% for plasma and 99.1% ± 0.59% for DBS.

Frequency of proviral DNA amplification from DBS

We also assessed the amplification contribution of proviral DNA in the TNA obtained from DBS using 8 samples with VLs ranging from 15,508 to 3,264,850 copies/ml. These 8 samples were run in the presence and absence of the RT-PCR MuLV enzyme. In the presence of the reverse transcription enzyme, the pol gene was successfully amplified in all eight samples, but only one sample with a higher VL (3,264,850 copies) could be amplified in the absence of the RT-PCR enzyme.

HIV-1 subtype analysis

Of the 64 newly obtained sequences, 49 were obtained from independent participants and the remaining 15 were obtained at different time points from these participants. Of the 49 samples, 36 were subtype A1 (73.5%), 4 were D (8.2%), 2 were C (4.1%), and 1 was G (2.0%) while 6 (12.2%) were recombinants: 4 CRF10_CD and 2 AD recombinants (Fig. 1).

Comparative analysis of cost and total hands-on time between the two assays using plasma and DBS samples

Table 5 describes the hands-on time and cost of the different steps involved in the HIVDR testing process, from sample collection to generating results. The cost included reagents and disposables based on 2011 U.S. dollars plus the cost for major equipment maintenance. Fixed costs, such as purchasing equipment and software, personnel, and other logistics, such as those of storage, transportation, and external quality assurance (EQA), were omitted. The cost of running the in-house assay using DBS specimens was $110. 05, that for the in-house assay using plasma specimens was $113.33, and that for the ViroSeq system using plasma specimens was $278.31. In addition, the in-house assay using plasma required the least hands-on time, ~16 h 30 min, compared to ~17 h 10 min for ViroSeq and ~24 h 20 min for the in-house assay using DBS, which required the longest hands-on time due to the manual extraction procedure.

Figure 1: Phylogenetic analysis showing correlation of plasma genotypes from the in-house assay and ViroSeq assay and DBS from the in-house assay.

IH, in-house; PL, plasma; VS, ViroSeq. The number at the node denotes bootstrap values of greater than 70%.

Table 5: Genotyping Cost analysis and estimated hands-on time [a]

Process	Steps	Assay-Sample Type					
		ViroSeq-Plasma		In-house Plasma		In-house DBS	
		Hands on time	Cost/test ($)	Hands on time	Cost/test ($)	Hands on time	Cost/test $
Sample preparation	Sample Collection (either DBS or Blood in EDTA) and plasma separation	6 hrs	6.00	6 hrs	6.00	10 min	1.00
Nucleic acid extraction	-RNA extraction-plasma -TNA extraction-DBS	3 hrs	150	1 hr	5.13	3 hrs	6.85
Amplification	-RT-PCR -One step RT-PCR -Nested PCR	5 hrs 20 min		4 hrs 3 hrs	13.96 5.12	4hrs 3hrs	13.96 5.12
Gel documentation	-Gel electrophoresis	1 hr		1 hr	19.22	1hr	19.22
Genotyping	-Amplicon purification -Sequencing	50 min 2hrs 30 min	90	30min 2hrs 30min	3.59 28.0	30min 2hrs 30min	3.59 28.0
	-Sequence amplicon purification -Sequence detection, visualization	1hr 30min 2hrs 30min	13.76 14.53	1hr 30 min 2hrs 30min	13.76 14.53	1hr 30 min 2hrs 30min	13.76 14.53
Sequence analysis	Sequence validation, interpretation and quality analysis	20 min	–	20	–	20	–
Equipment maintenance	ABI 3100 & thermocyclers, bio-safety cabinets.	–	4.02	–	4.02	–	4.02
Total		17hrs 10 min	278.31	16 hr 30 min	113.33	24 hrs 20 min	110.05

[a] The cost was estimated based on U.S. dollars at the 2011 market values. The estimated costs also included the costs to run control specimens in each run and equipment maintenance but excluded fixed costs, personnel costs, and other logistics, such as storage, transportation, and enrollment in EQA programs, which are vital for the quality-assured genotyping results

DISCUSSION

We observed excellent concordance of DRM detections in plasma and DBS by the in-house genotyping assay compared to the ViroSeq assay and confirmed the previous report on the excellent performance of this assay (32, 33). The overall sensitivity and specificity of the in-house assay in detecting DRMs were 98.29% and 99.97% for plasma and 96.54% and 99.97% for DBS, respectively. The findings from this study affirm previous reports that DBS specimens offer an excellent alternative sample type for HIV genotyping for HIVDR tests (13, 29, 32, 42–46). This study further demonstrates their utility and feasibility in a resource-limited setting for use in HIVDR monitoring and surveillance combined with a less costly and subtype-independent in-house genotyping assay. The overall genotyping efficiency rate for the in-house assay was 94% compared to 78% by the ViroSeq system. The relatively higher genotyping rate for the in-house assay on plasma specimens may be attributed to the shorter fragment target (1.1 versus 1.8 kb) as well as the inclusion of a nested PCR step. However, this shorter amplicon has no effect on the interpretation of the known clinically relevant DRMs described in the IAS 2011 mutation list for the protease and RT regions (36).The overall genotyping rate for DBS specimens using the in-house assay was moderate (76%). However, when only those DBS specimens with VLs of ≥1,000 copies/ml were considered according to the WHO recommendation for ART patient monitoring (47), the genotyping rate was greatly improved (89.5%). The lower-than-expected DBS genotyping rate might result in the lower input for TNA extraction in the current study, since we used only two 6-mm discs, which are equivalent to 32 µl of whole blood. A previous study by Masciotra et al. (13) showed that TNA amplification from lower-VL samples can be achieved from DBS by using 2 complete spots (about 100 µl). This was, however, not feasible in this analysis due to the depletion of some of the samples. In fact, the moderate success rate despite lower initial sample input may be a reflection of a higher sensitivity of the in-house assay. Apart from the lower initial input, DBS genotyping efficiency may also be dependent on the storage conditions; in this case, the DBS samples had been stored for a period of up to 4 years under optimal conditions of −30°C. This demonstrates the integrity of the DBS samples in preserving the viral genetic materials for a long period under optimal storage conditions and is consistent with the findings from the work of Masciotra et al. (13). This is vital in HIVDR monitoring surveys in resource-limited settings where testing is usually performed either in batched samples at the centralized

genotyping laboratories within the country or in regional laboratories outside the country in some situations.

The sensitivity and specificity of the in-house assay in detecting DRMs were highly concordant with those obtained in the ViroSeq system for both plasma and DBS. The discordant DRMs detected between the in-house and ViroSeq on both DBS and plasma were mainly caused by mixture bases at the DRM sites. These differences, however, were not of clinical significance, as genotypic algorithms infer the presence of resistance for a synonymous mixture mutation in the same way as they do for a complete mutation.

Another important requirement in method validation is the ability to produce accurate results within a run or between runs under similar assay conditions. The in-house assay assessed using the WHO guidelines for genotyping method validation demonstrated both good intra- and inter-run comparisons as well as excellent performance in proficiency test panels for both plasma and DBS. This further confirmed a possible concordance of results if this assay is adopted for use in other laboratories in resource-limited settings. In fact, the assay has been implemented in the Ethiopian national DR laboratory and the laboratory has been accredited by the WHO ResNet group as a National DR Laboratory (NDRL) (http://www.who.int/hiv/topics/drugresistance/technical_information/en/index.html).

The high concordance of the DRMs detected by the ViroSeq commercial assay and in-house assay (both plasma and DBS) suggests the utility of this assay in both HIVDR surveillance and monitoring. Twenty-five of the samples used in the analysis were obtained from mothers experiencing virological failures (plasma VLs of ≥1,000 copies/ml) in the KiBS trial of prevention of mother-to-child transmission (PMTCT). The HIVDR status of these mothers had been assessed in real time during the trial using the ViroSeq system; thus, confirmation of the DRM detection using the in-house assay on both plasma and DBS suggests the clinical utility of this assay as an HIVDR monitoring tool using both sample types. However, due to the contribution of proviral DNA from peripheral blood mononuclear cells (PBMCs) in DBS, DBS is not recommended for use in long-term ART-experienced patients, as the detected DRMs may not be a full reflection of the circulating viruses but may reflect archived ones (31). Archived viruses in the DNA have been reported to have different mutation patterns from those of circulating viruses, especially with ART-experienced persons (25, 29, 45). As

a result, genotyping from DBS which contain both the proviral DNA and free circulating RNA viruses is expected to give mutation patterns different from those of plasma in ART-experienced persons in the case of the presence of archived mutations. In this study, we observed minimal proviral contributions with only two discordant major mutations: one present in DBS but missing in plasma and vice versa. Of interest was the low frequency of proviral DNA amplification; only 1 of the 8 samples was amplified in the absence of an RT enzyme, with the amplified sample having a very high VL of >3.6 million copies/ml. This may suggest that genotyping from DBS involves mainly the free circulating viral RNA rather than the archived DNA provirus. However, the use of DBS in long-term ART-experienced patients should be made with caution, as the samples in this analysis were obtained from patients who were on ART for <1 year.

The ability to genotype multiple HIV-1 subtypes and CRFs is essential for any genotyping assay in sub-Saharan Africa where multiple subtypes and CRFs exist (32, 48–50). This is specifically important since the two FDA-approved assays were developed and evaluated for use with subtype B viruses (23). Previous studies using the in-house assay have shown its suitability as a broadly sensitive genotyping assay which was able to genotype HIV subtypes C, B, CRF 01 AE and 02 AG, A, and CRF 07 and 08 BC (32, 33). From this analysis, we further affirm its broad subtype sensitivity, especially for subtypes A and D, which are also the predominant HIV strains in Kenya. It is important to note that the ViroSeq system also gave a satisfactory performance in genotyping the different virus strains in this study. However, the lower DBS sensitivity reported before (13) may restrict its utility in resource-limited settings for HIVDR monitoring surveys due to logistical constraints for collecting plasma specimens.

One of the reasons that the HIVDR genotyping test has not become a routine service is the high cost associated with HIVDR monitoring. In this study, we performed analysis on the cost associated with the two assays using the 2011 market values in U.S. dollars. Cost assessments of the in-house assay against the ViroSeq system showed that genotyping of DBS or plasma with the in-house assay could reduce the HIVDR testing cost by about 60%.

This study had some limitations. First, our sample size was relatively small, although it met the calculated sample size for our study purpose. Another limitation of this study is the DBS collection procedure, which was inherited

from the original study design, for which the DBS from infants had not been collected by either finger or heel pricks. However, studies using DBS collected from infants by heel/finger pricks are under way, which will add the needed data for the feasibility of using DBS collected by heel/finger pricks for HIVDR testing. Despite the limitations of the study, we believe that there are several strengths. (i) The use of the recently developed WHO guidelines in the validation process not only increases the confidence of the performance of the assay but also serves to ease the adoption by other laboratories in resource-limited settings. (ii) This validation was conducted under real field setup conditions, and it was a true reflection of how this assay could be performed in resource-limited settings. (iii) The implementation of the in-house assay in another resource-limited country and further attainment of WHO ResNet accreditation as mentioned above are another testimony that if vigorous implementation procedures are followed, then the low-cost in-house assay, like the one that we validated here, can be successfully implemented in resource-limited settings. Other considerations like having well-trained, qualified, and competent personnel and meeting the minimal infrastructure requirement as well as participation in external proficiency programs should also be in place.

In conclusion, this study demonstrates the excellent performance of a lower-cost in-house genotyping assay for both plasma and DBS specimens for use in HIVDR surveillance and ART patient monitoring. This assay would be particularly appropriate for use in resource-limited settings with DBS specimens. These findings are of particular interest due to the increased need for HIVDR genotyping in resource-limited settings in the era of increasing demand for ARV usage in treating HIV-infected patients as well as treatment for prevention.

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Chapter 14

Affordable HIV drug resistance testing for monitoring antiretroviral therapy in sub-Saharan Africa: perspectives and challenges

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SUMMARY

Increased provision of antiretroviral therapy in sub-Saharan Africa has led to a growing number of patients with therapy failure and acquired drug-resistant HIV, driving the demand for more costly further lines of antiretroviral therapy. In conjunction with accelerated access to viral load monitoring, feasible and affordable technologies to detect drug-resistant HIV could help maximise the durability and rational use of available drug regimens. Potential low-cost technologies include in-house Sanger and next-generation sequencing in centralised laboratories, and point mutation assays and genotype-free systems that predict response to antiretroviral therapy at point-of-care. Strengthening of centralised high-throughput laboratories, including efficient systems for sample referral and results delivery, will increase economies-of-scale while reducing costs. Access barriers can be mitigated by standardisation of in-house assays into commercial kits, use of polyvalent instruments, and adopting price-reducing strategies. A stepwise rollout approach should improve feasibility, prioritising WHO-recommended population-based surveillance and management of complex patient categories, such as patients failing protease inhibitor-based antiretroviral therapy. Implementation research, adaptations of existing WHO guidance, and political commitment, will be key to support the appropriate investments and policy changes. In this Personal View, we discuss the potential role of HIV drug resistance testing for population-based surveillance and individual patient management in sub-Saharan Africa. We review the strengths and challenges of promising low-cost technologies and how they can be implemented.

INTRODUCTION

Unparalleled antiretroviral scale-up efforts in sub-Saharan Africa, the region most affected by the HIV epidemic, resulted in approximately 11 million people receiving antiretroviral therapy (ART) by 2015.[1] To sustain ART success in Africa, investments in the global response and national policies need to shift towards sustaining of viral suppression to reduce HIV transmission and prevent large-scale HIV drug resistance. The global 90-90-90 treatment goals for 2020, launched by UNAIDS, focus on optimization of ART delivery, including accelerating access to viral load testing to monitor treatment effectiveness.[2]

Nonetheless, a substantial proportion of adults and children who receive first-line ART, which contains two nucleoside reverse-transcriptase inhibitors (NRTIs) plus a non-NRTI (NNRTI), will develop virological failure during the first 5 years.[3, 4] Of these patients, 70–90% acquire drug-resistant HIV[5, 6, 7, 8] which limits subsequent treatment options. Moreover, since the ART scale-up, levels of pre-treatment drug resistance have been increasing in the region,[9, 10, 11] impairing initial responses to ART.[12, 13] The number of people on second-line ART in sub-Saharan Africa is fewer than 1 million now, but is expected to grow to around 4–6 million people by 2030, with implementation of universal viral load monitoring (which will enhance diagnostic abilities to diagnose failure), comprising up to 20% of all those on ART.[14] These developments will also drive the demand for third-line and further therapies.

Therefore, an emerging challenge in low-income and middle-income countries (LMICs), is how to manage the growing numbers of patients in need of second, third, or even further lines of therapy, where the availability of antiretroviral drugs, diagnostics, and clinical expertise is very limited. By contrast with high-income countries,[15, 16] genotypic resistance testing (GRT) to guide selection of initial and subsequent ART is generally not feasible in LMICs, because of limited laboratory capacity and high test costs. Access to new antiretroviral drugs—including second-generation protease inhibitors and integrase inhibitors—remains limited in LMICs. The cost for proposed third-line drugs—raltegravir, darunavir and etravirine—is prohibitive at US$1800 per year, which is nearly six times more expensive than protease inhibitor-based second-line and up to 15 times more expensive than NNRTI-based first-line therapy.[17] For children, drug options beyond first-line are even more limited.[18]

Promising new ART drugs such as dolutegravir and tenofovir-alafenamide have obtained voluntary licences in several LMICs. Dolutegravir has a high genetic barrier to resistance and could potentially reduce resistance development.[19] However, experience with dolutegravir is limited and close resistance monitoring will be required when it is implemented on a large scale and in a range of settings and patient populations. Thus, the existing antiretrovirals are expected to remain highly relevant for the foreseeable future. To maximize durability of available ART options, affordable and feasible diagnostic tools are needed to assess drug-resistant HIV and improve rational drug selection after ART failure.

In this Personal View, we discuss the potential role of HIV drug resistance testing for population-based surveillance and individual patient management in sub-Saharan Africa, in the context of evolving ART practices and growing demand. We review the strengths and challenges of promising low-cost technologies, and explore how they can be implemented.

Evolving policies for ART monitoring in LMICs

Over the past decade as access to affordable technologies improved in LMICs, WHO guidance has evolved to recommend phasing in of routine viral load monitoring of ART, to replace CD4 cell counts (figure 1).[18, 20] Accelerated access to viral load monitoring will increase the number of patients diagnosed with ART failure who require a regimen switch.[14] The global Diagnostics Access Initiative is committed to building the required robust, sustainable laboratory capacity to meet the 90-90-90 goals.[21]

Several barriers limit expanded use of GRT in LMICs, including high capital investment and test costs, limited molecular laboratory infrastructure, lack of skilled staff, and need for complex cold-chain sample logistics. At the population level, WHO recommends that countries implement a national strategy to assess and prevent HIV drug resistance.[22] In the past decade, GRT laboratory capacity in LMICs has been strengthened, mostly to facilitate the WHO-recommended surveys (figure 2, tableS1). Despite striking progress, many countries have been struggling to implement these surveys, mainly because of lack of funds, budget prioritization, and the complexity of survey protocols.[8] The 2015 WHO guidelines generally do not recommend the use of individual GRT, except for optimizing of third-line therapy (after failure of darunavir-based second-line).[18] Some countries, such as Botswana, Kenya, South Africa, Uganda, and Zambia, have indeed

cautiously started to provide GRT for patients with second-line failure, to identify individuals who have protease inhibitor-resistance and to reserve expensive third-line drugs for those with the greatest need.[23, 24, 25]

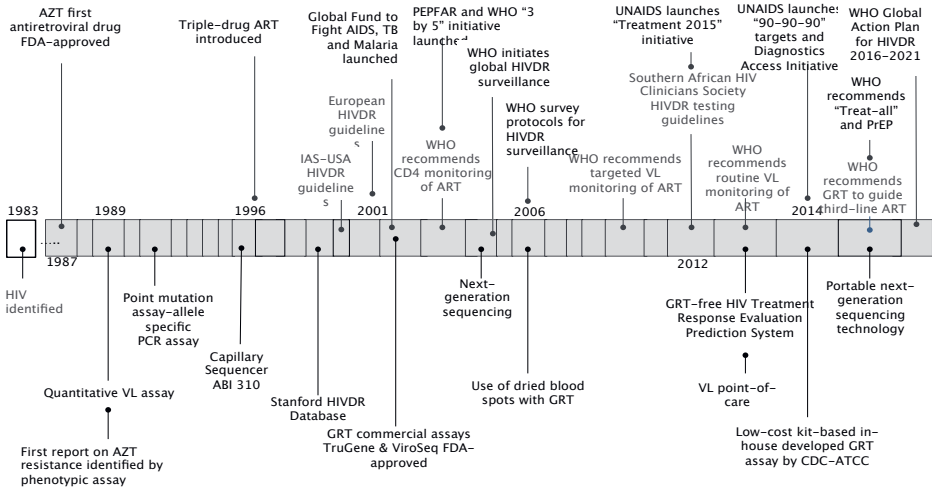

Figure 1: Timeline of anti-retroviral therapy monitoring technologies and policies in sub-Saharan Africa

The fast development of new technologies to monitor ART effectiveness contrasts with the slow adoption of ART monitoring strategies in LMICs. ART=antiretroviral therapy. ATCC=American Type Culture Collection. CDC=US Centers for Disease Control and Prevention. FDA=US Food and Drug Administration. GRT=genotypic resistance test. HIVDR=HIV drug resistance. PEPFAR=US President's Emergency Plan for AIDS relief. PrEP=pre-exposure prophylaxis. UNAIDS=Joint UN programme on HIV/AIDS. VL=viral load.

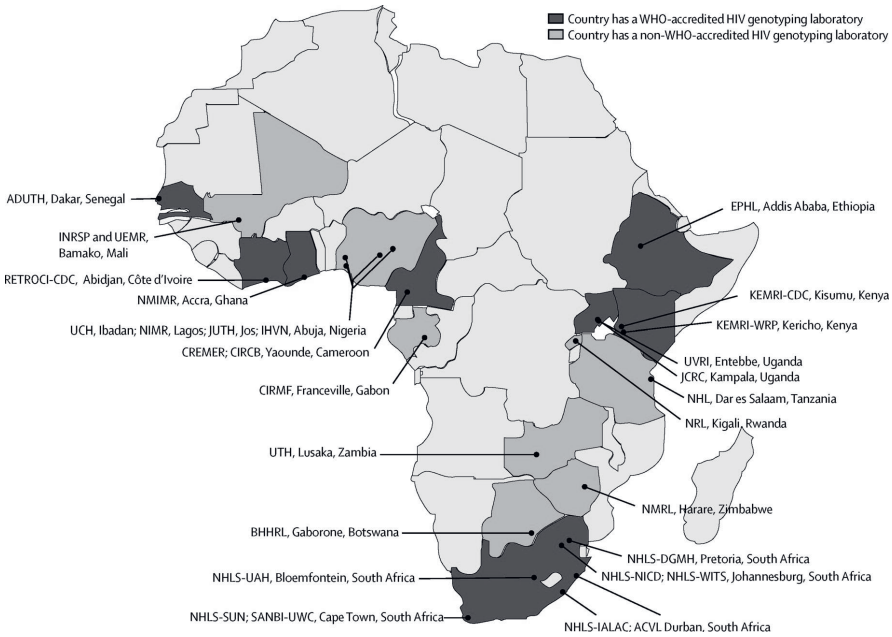

Figure 2: Laboratory capacity for HIV genotyping in sub-Saharan Africa Acronyms are explained in tableS1.

Potential benefits of HIV drug resistance testing for individual patient management

In high-income countries, individual GRT with expert advice leads to improved virological response in adults and children with ART failure and in previously untreated patients,[26, 27, 28] and is cost-effective,[29, 30] which informed guidelines to use GRT in guiding initial and subsequent ART regimens.[15, 16]

The use of GRT in patients with virological failure in LMICs has two potential benefits. First, GRT can improve the specificity of failure detection—ie, avert switching in patients who do not carry clinically relevant resistance mutations, thus preserving more costly next regimens for those who need it. Reports from the sub-Saharan African region suggest that 10–30% of first-line failure cases do not have any NRTI or NNRTI-associated resistance mutations,[5, 31] and that up to 70–90% second-line failure cases do not have any major protease inhibitor-associated resistance mutations.[32, 33, 34] Second, individual GRT profiles could guide selection of the most effective drug combinations. Specifically, GRT could help construct second-line regimens (in children who received protease

inhibitor-based first-line) as well as third-line or salvage regimens, allowing the re-use of cheaper drugs where possible. For example, the multicountry MULTI-OCTAVE trial (NCT01641367) is ongoing to assess the use of novel antiretrovirals and GRT to optimize treatment after virological failure.

Cost-effectiveness of individual GRT in LMICs has been debated. In patients failing first-line ART, two studies used mathematical models based on South African data to compare the use of GRT to guide first-line to second-line switches to a blanket switch based only on routine viral load monitoring.[35, 36] Levison and colleagues[35] estimated that GRT is cost-effective at $900 per life-year saved and it would be cost-saving if the test cost was less than $100. Rosen and colleagues[36] estimated that incorporation of GRT into national ART guidelines would be cost-neutral. A study[37] based on data from Zimbabwe, however, estimated that GRT was not cost-effective, even at very low test cost. In patients failing second-line ART, a study in South Africa suggested that GRT to guide decisions for third-line therapy could improve survival and be cost-effective, whereas a public health approach (ie, switching all second-line failures to third-line treatments) was deemed unaffordable.[38]

Promising low-cost technologies for HIV drug resistance testing in LMICs

GRT assays are tests that detect specific amino acid changes resulting from mutations within the portion of the viral genome that the drug targets. GRT assays can be classified as point mutation assays (PMAs) and sequencing-based assays; and sequencing-based assays are further classified into Sanger and next-generation sequencing (NGS). Several technology developments have occurred that could advance HIV drug resistance diagnostics in LMICs (panel 1).

Panel 1: Strengths and challenges of low-cost HIV drug resistance technologies

In-house Sanger sequencing

Strengths

- Widely validated, some adapted into kit-based assays
- Broad subtype based
- Most commonly available technology in Africa
- Use with dried blood spots

Weaknesses

- Limited potential for automation hence complex and labour intensive
- Not suited for parallel testing
- Cannot detect minor variants (<20%)
- High capital cost

Next-generation sequencing

Strengths

- Cost-saving through sample pooling and parallel testing
- High sensitivity for minor variants
- Potential for use with widely validated broad-subtype based primers
- Potential for use with dried blood spots

Weaknesses

- Complex workflow, labor intensive
- Requirement of specialized facilities
- PCR errors can lead to overestimating resistance
- Possible sequence read problems in homopolymer regions (Ion-torrent) or unequal sequencing coverage (Illumina Miseq)
- Data analysis complex and costly
- Requires wide validation
- High capital cost

Point mutation assays

Strengths

- High sensitivity for minor variants
- Potential for use with widely validated broad subtype-based primers

Weaknesses

- Limited ability for multiplexing hence complex, laborious and increased cost
- Not commercially available
- Requires wide validation

Genotype-free prediction systems

Strengths

- Low capital and running costs (mainly internet infrastructure needs)
- High accuracy in prediction of optimum regimens
- Potential for use at point-of-care

Weaknesses

- Requires pre-switch CD4 and viral load results
- Could result in unnecessary regimen switch (in case of absence of drug-resistance mutations)
- Requires wide validation

Sanger sequencing

Sanger sequencing is based on the sequence terminating method to analyze a single DNA fragment. To date, only one assay for the polymerase gene (protease and reverse-transcriptase) has been FDA-approved (ViroSeq, Abbott, Abbott Park, IL, USA).[39] To reduce test costs, many research laboratories have developed in-house assays with comparable performance.[40, 41, 42, 43] Costs can be reduced by use of open-source reagents (which limits patent-related costs), reducing number of primers, single-step amplification,[40, 41, 42, 43] collective bargaining with suppliers (SATuRN/Life Technologies assay, ThermoFisher Scientific, Waltham, MA, USA[41, 44]), partnering with nonprofit manufacturers (Centers for Disease Control and Prevention [CDC]/American Type Culture Collection [ATCC], Manassas, VI, USA/ThermoFisher assay, ThermoFisher Scientific[43, 45]), sequencing only reverse-transcriptase for first-line failures (ART-A assay[40]), and sample pooling.[46] Some assays also have broad HIV-1 subtype coverage and can be used with dried blood spots with reasonable (1000–5000 HIV-RNA copies per mL) amplification sensitivity.[47, 48] Moreover, some assays have been developed into kit-based commercial assays (CDC/ATCC/ThermoFisher[43, 45] and SATuRN/Life Technologies assays[41, 44]), which may enhance their scalability for use in laboratories with Sanger sequencing equipment (figure 2, table S1). Present test prices (without labour costs) range from $155 to $276 for the commercial assays, and from $47·50 to $155 for in-house assays (table S2).

With simplified Sanger assays available, we anticipate that Sanger-based GRT will continue to play a substantial role in the short-term, because most alternative technologies will require time for validations and effective rollout. However, Sanger GRT is limited in throughput and needs sophisticated and costly equipment, which is only available in centralized laboratories (panel 1).

Next-generation sequencing

The development of robust NGS allows parallel sequencing of millions of DNA fragments, which can be exploited for HIV drug-resistance testing. Although equipment costs are still high (>$100 000), prices will reduce as the technology simplifies. Current commercially available lower-cost devices are Ion-Torrent PGM (ThermoFisher Scientific, $50 000) and Illumina MiSeq (Illumina, San Diego, CA, USA, $99 000).[49] The cost per run is generally high, but low cost per test and higher levels of automation can be achieved through multiplex sequencing, where many individually primer-barcoded samples are pooled using 24-multiplex

(Illumina MiSeq50) or 48-multiplex (Roche 454[51]) systems. Multiplex sequencing is a potentially affordable alternative to Sanger in centralized high-throughput laboratories. Moreover, the potential for full-genome sequencing with minimal additional cost could make NGS a preferred option for evaluation of resistance in patients on protease inhibitor-based therapy, because polymorphisms in group-specific antigen (gag) protein and envelope (env) gene regions could induce protease inhibitor-resistance.[52, 53] Full-genome sequencing also minimizes the need for separate assays for the integrase gene region,[54] since the use of integrase inhibitors is anticipated to increase. Further advantages of NGS include the opportunities for simultaneous detection of other pathogens, and use with dried blood spots[55] and broad-subtype primers.[49]

Limitations of NGS are the high-level expertise and expensive bioinformatics support required for data analysis.[56] Moreover, pyrosequencing methods (eg, Ion-Torrent PGM) are prone to insertion–deletion errors at homopolymer regions,[57, 58] and reversible-terminator methods (Illumina MiSeq) are prone to substitution errors and unequal sequencing coverage.[57, 58, 59] Regional bioinformatics hubs to reduce data analysis costs are needed to increase the usefulness of these assays in LMICs (panel 1).

Point mutation assays

PMAs, which identify only specific sequence changes, are appealing because they are low cost, have high sensitivity for minor mutation variants and could be used in a point-of-care test platform.[60] Several types of PMA exist, key of which are the allele-specific primer or probe extensions assays with fluorescent or colorimetric detection mechanisms.[61, 62, 63, 64, 65, 66, 67, 68, 69] Upcoming low-cost technologies include Luminex-based microarray assays,[61] allele-specific PCR based PANDAA,[68] and GeneXpert[70] assays (Cepheid, Sunnyvale, CA, USA) and a modified paper-based detection oligonucleotide ligation assay.[69] A PMA that can detect a set of six resistance mutations in reverse-transcriptase (Lys103Asn, Val106Met, Tyr181Cys, Gly190Ala, Lys65Arg and Met184Val) successfully detected resistance in 98·8% of patients failing first-line and 61·2% of previously untreated patients.[60] The main problem with the PMA technique is the substantial HIV-1 sequence variability at and surrounding each drug resistance mutation target, which means multiple experiments are needed per target site. The requirement for multiple tests per site increases the costs of PMAs, especially without affordable multiplexing assays. Therefore, development and validation of low-cost multiplex PMAs might lag behind current clinical practice (panel 1).

Genotype-free prediction systems

An alternative approach is provided by machine-learning methods, which use computational models to provide predictions for virological response to ART on the basis of large databases of ART-treated patients. They evaluate and rank a set of suitable treatment regimens to select the most suitable drug combinations for a given patient. Genotype-free models have been developed that predict a given patient's virological response by means of only demographical and clinical data, such as most recent viral load and CD4 cell count, and previous treatment history.[71] The HIV Treatment Response Prediction System (HIV-TRePS) had an overall accuracy of 78% in prediction of virological response in independent testing,[71] compared with 57–60% achieved by GRT with rules-based interpretation for the same cases. HIV-TRePS, available as a free online service, has the potential to enhance clinical decision-making of ART providers with relatively little training (panel 1).

Approaches to implementing HIV drug-resistance testing in LMICs

Prioritizing patient categories for HIV drug-resistance testing

Although there is potential to simplify the provision and reduce the cost of GRT, a stepwise rollout approach is still expected to be most feasible. With this strategy, predefined patient categories that will probably benefit most should be prioritized. This approach will also allow the logistical and technical laboratory capacity to be firmly established before scaling up services to wider patient categories (panel 2). However, its success will be dependent on local conditions, available resources and future WHO normative guidance.

Panel 2: Proposed stepwise rollout approach to HIV drug-resistance testing in sub-Saharan Africa

1. Enhancing population-based surveillance of HIV drug resistance

- Implementation of the WHO-recommended strategy22 in a routine manner as part of national ART programmes will help improve programme functioning, thereby mitigating HIV drug resistance at the population level.

2. Patients with ART failure (specifically children with failure of first-line or second-line PI-based ART, and adults with failure of second-line PI-based or further lines of ART)

- Individual GRT can guide decision-making to either maintain current therapy (if no documented PI-resistance) or optimise next-line regimens (if documented PI-resistance), combining recycled or new drugs. For children, this strategy could include either an NNRTI (if no NNRTI-resistance and if >3 years of age) or an INSTI (either raltegravir, or dolutegravir if >12 years of age), and for adults darunavir with raltegravir or dolutegravir, with or without optimised NRTIs. The 2015 WHO guidelines recommend use of GRT profiles to optimize third-line line ART.18

3. Patients before starting first-line NNRTI-based ART

- After prior ART exposure. Presence of resistance due to prior use of ART, pre-exposure or post-exposure or mother-to-child prophylaxis, might require individualized first-line treatment—ie, optimized NRTI-backbone or regimens based on PIs or INSTIs.

- All patients. Routine individual GRT could be used in settings where a high proportion (threshold not well-defined, possibly >15%) of pre-ART patients have NRTI-resistant or NNRTI-resistant viruses, to identify individuals requiring individualized first-line treatment—ie, optimized NRTI-backbone or a PI-based or INSTI-based regimen. The usefulness of this strategy will depend on the cost-effectiveness of routine GRT with NNRTI-based first-line as opposed to changing standard first-line to, for example, a dolutegravir-based regimen.

ART=antiretroviral therapy. PI=protease inhibitor. NRTI=nucleoside reverse-transcriptase inhibitor. NNRTI=non-NRTI. GRT=genotypic resistant testing. INSTI=integrase inhibitors.

Centralized laboratory services

High levels of technical expertise and infrastructure are required for GRT and appropriate point-of-care tests are lacking within the current landscape. Therefore, we believe the most feasible approach to expand access to GRT in sub-Saharan Africa is the adoption of centralized high-throughput testing facilities that are strategically situated to serve the country or region, both for population-based surveillance and patient management (figure 2, table S1). It will be easier to solve the challenges of sample referral and results delivery, as there is already substantial experience with this issue, than to solve the challenge of decentralization of GRT with available technologies. Decentralizing GRT at or near point-of-care with moderate anticipated test volumes would probably be impractical and overly expensive.

GRT for patient management is only useful if available in a clinically relevant timeframe. Therefore, such testing hubs should be connected to remote clinical sites through efficient systems for sample referral. Perhaps the greatest challenge to centralized testing is the need for short turnaround times for test results and high levels of data accountability. However, developments in mobile health connectivity applications will make implementation of quality-controlled data information systems, such as online sample tracking systems feasible, for rapid distribution of test results (with expert review and interpretation) to health care providers.[72] Dried blood spots could become the preferred sample type because they are easily shipped and stored,[47, 48] especially when they have imprinted barcodes or microchips that allow for real-time tracking and tracing.

Candidate laboratories to provide centralized GRT services would ideally build on existing capacities, including WHO-designated HIV genotyping laboratories, national reference laboratories and, potentially, high-volume viral load laboratories (figure 2, table S1). Centralization of laboratory testing will be influenced by available infrastructure, test volumes, quality systems, and level of expertise, in each country. International quality standards should be maintained through rigorous training programmes and links to external quality assurance schemes. Additional training on how to interpret GRT results will be needed to support clinical management. Once established, these central hubs will not only allow the scale-up of HIV GRT, but also provide opportunities to support testing needs for detection of other common pathogens and antimicrobial resistance (such as tuberculosis or hepatitis B and C). Moreover, these hubs allow for establishment of national databases for HIV drug resistance to help shape national ART policies.

Expanding access

Several additional approaches would help overcome existing barriers to the use of GRT. First, commercialization of in-house sequencing assays; standardization into kits will allow scalability, simplification, and reduction of reagent costs of GRT assays. Examples include the CDC/ATCC/ThermoFisher[43, 45] and SATuRN/Life Technologies assays.[41, 44] Adaptation of WHO ART guidelines will be required to expand HIV drug resistance testing in LMICs, similar to evolving policies of viral load monitoring. Current demands for HIV drug resistance tests in LMICs could be commercially unattractive to suppliers with dedicated HIV GRT systems. Molecular systems should be used that are polyvalent for HIV GRT as well as antimicrobial drug resistance, tuberculosis, viral hepatitis, and other common pathogens—

for example, the GeneXpert platform. Development and empowerment of regional centres of excellence to rapidly conduct validation studies of new diagnostics and devices could help generate data on technology performance to inform regulatory processes and technology adoption. Networks of centres of excellence under WHO, US CDCs or the African Society for Laboratory Medicine could provide good platforms to support multi-country validation studies.

Conclusions and future directions

With life-saving treatment now a reality for millions of HIV-infected Africans, the next challenge is to ensure life-long provision of effective ART. In conjunction with wider access to viral load monitoring, affordable technologies to detect drug-resistant HIV can help preserve available drug regimens for as long as possible. In this Personal View, we argue that the implementation of low-cost approaches to HIV drug resistance testing could greatly advance ART decision-making both for individual patients and public policy. Clinical benefits for patients include prevention of premature switches to costly regimens—if clinically relevant mutations are not detected—and improvement of virological responses through selection of optimal drug combinations for the increasing numbers of children and adults experiencing ART failure. Continuous resistance surveillance, using aggregated data from individual patients and population-based surveys, will be key to improve ART programmes and to adapt ART guidelines as necessary.

Advances in HIV drug-resistance testing have the potential to lower costs and achieve increased access in LMICs. We believe the most feasible approach to expand access in resource-constrained environments is to adopt centralized high-throughput testing facilities. While in the short-term in-house Sanger-based GRT will remain the predominant technology, NGS has great potential for the medium-to-long term, offering the possibility of multiplexing very large numbers of samples in a single sequencing reaction, lowering costs even further in high-throughput laboratories. Improvements in existing or new point-of-care technologies could provide the opportunity to decentralize GRT. For example, improved PMA technology could encourage decentralization especially through integration into existing nucleic-acid point-of-care systems such as the widely accessible GeneXpert platform. Genotype-free prediction systems through free online services can help ART providers at point-of-care to select optimum therapy from locally available options, offering a practical alternative to blood-based technologies. Each of these technologies will require comprehensive

clinical evaluations in various populations to definitively demonstrate their utility and cost-effectiveness. The table outlines the potential specifications of an ideal drug-resistance test for use in sub-Saharan Africa.

Table Potential specifications of an ideal HIV drug resistance test for use in sub-Saharan Africa

Parameter	Ideal characteristics
Assay characteristics	
Sample collection method	Dried blood spots
Subtype coverage	Broad subtype-based for major HIV-1 group M viruses
Cost per test	≤ USD 50
Number of mutations*	At least all major NNRTI, NRTI, PI and INSTI † mutations
Consumables per result	Minimal, open access to consumables and readily available from suppliers
Reagent characteristics	Minimal logistics for refrigeration, and readily available from suppliers
Instrument characteristics	
Technology set-up	Open-access system
Instrument cost±	USD 20,000-50,000
Complexity	Minimal complexity preferably fully automated
Training	Medium level technical training
Analysis method	Minimal complexity preferably fully automated
Performance characteristics	
Time to results	≤14 days
Amplification sensitivity	1,000-5,000 cps/ml
Sensitivity for low abundance species	≤20%
Registered as in vitro diagnostics	Minimum: WHO approved

INSTI, integrase strand transfer inhibitor; NRTI, nucleoside reverse transcriptase inhibitor; NNRTI, non-NRTI; PI, protease inhibitor; * In patients failing first-line NNRTI-based therapy, a test targeting only NNRTI and NRTI mutations may be appropriate.
†For settings introducing integrase inhibitors.
±Preferably less than USD 20,000 for point-of-care testing and less than USD 50,000 for centralized testing.

Stepwise rollout strategies with prioritized patient categories will improve feasibility (panel 2). Test access barriers can be mitigated by standardization of in-house GRT assays into commercial kits, using polyvalent instruments and providing cooperative licensing strategies to stimulate generic manufacturers. The strengthening of centralized high-throughput laboratory testing hubs, including efficient systems for sample referral and results distribution, will be key to increase the economies-of-scale while greatly reducing costs. A lot can be learned from countries like Botswana, South Africa, Uganda, and others that have started implementation of routine GRT into their ART programmes.

Last, commitment and leadership of global policy makers and national governments will be key to the success of any of these efforts, as illustrated by previous initiatives to improve access to ART and viral load in LMICs. The Global Health Action Plan for HIV Drug Resistance (2016–2021),[73] currently being developed by WHO and key partners, will provide an important stimulus towards a coordinated and resourced response in LMICs.

Contributors

All authors conceptualized the paper. SCI and RLH wrote the first draft of the paper. All authors commented on and contributed to subsequent versions, and read and approved the final paper.

Declarations of interest

We declare no competing interests.

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APPENDIX

Table S1: Current laboratories for HIV genotypic resistance testing in sub-Saharan Africa

Country	Acronym	Institution
Botswana	BHHRL	Botswana Harvard HIV Reference Laboratory, Gaborone
Cameroon	CREMER	Centre de Recherche en Maladies Emergentes et Ré-émergentes, Yaounde
	CIRCB	Centre International de Référence Chantal Biya, Yaounde
Cote d'Ivoire	RETROCI-CDC	Retrovirus Cote d'Ivoire-US Centers for Disease Control and Prevention, Abidjan
Ethiopia	EPHL	Ethiopia Public Health Laboratory, Addis Ababa
Ghana	NMIMR	Noguchi Memorial Institute for Medical Research, Accra
Gabon	CIRMF	Centre International de Recherches Medicales de Franceville, Franceville
Kenya	KEMRI-CDC	Kenya Medical Research Institute-Centers for Disease Control HIV Research Laboratory, Kisumu
	KEMRI-WRP	Kenya Medical Research Institute-Walter Reed Project, Kericho
Mali	UEMR	Unité d'Epidémiologie Moléculaire de la Résistance du VIH aux ARV, SEREFO, Bamako
	INRSP	Institute National de la Recherche en Santé Publique, Bamako
Nigeria	IHVN	Institute of Human Virology Nigeria, Abuja
	JUTH	Jos University Teaching Hospital, Jos
	UCH	University College Hospital, Ibadan
	NIMR	Nigerian Institute of Medical Research, Lagos
Rwanda	NRL	National Reference Laboratory, Kigali
Senegal	ADUTH	Laboratory of Bacteriology Virology-Aristide Le Dantec University Teaching Hospital, Dakar
South Africa	ACVL	Africa Center Virology Laboratory, University of Kwazulu Natal, Durban
	NHLS-NICD	National Health Laboratory Services, National Institute for Communicable Diseases, AIDS Virus Research Unit Johannesburg
	NHLS-WITS	National Health Laboratory Services, University of the Witswatersrand, Clinical Laboratory Services, Johannesburg
	NHLS-SUN	National Health Laboratory Services, Tygerberg Hospital, Stellenbosch University, Cape Town
	NHLS-IALAC	National Health Laboratory Services, Inkhosi Albert Luthuli Academic Hospital, Durban
	NHLS-UAH	National Health Laboratory Services, Universitas Academic Hospital, Bloemfontein
	NHLS-DGM	National Health Laboratory Services, Dr George Mukhari Hospital, Pretoria
	SANBI-UWC	South African National Bioinformatics Institute, University of Western Cape, Cape Town
Uganda	JCRC	Joint Clinical Research Centre, Center for AIDS Research, Kampala
	UVRI	Uganda Virus Research Institute, Medical Research Council, Entebbe
Tanzania	NHL	National Health Laboratory, Quality Assurance and Training Center Dar es salaam, Tanzania
Zambia	UTH	University Teaching Hospital, Lusaka
Zimbabwe	NMRL	National Medical Reference Laboratory, Harare

Table S2: Current prices of HIV genotypic resistance tests provided by laboratories in sub-Saharan Africa

Country	Institution	City	WHO designation	Assay	Specimen type	Unit cost (USD)
Cameroon	CREMER	Yaoundé	National	[1]ViroSeq	Plasma	207
					DBS	156
	CIRCB	Yaoundé	_	[2]ViroSeq	Plasma	276
				[2]In-house	Plasma	117
Cote d'Ivoire	RETROCI-CDC	Abidjan	National	[1]In-house [1]	Plasma	87
Ethiopia	EPHL	Addis Ababa	National	[1]In-house [2]	Plasma	110
					DBS	75
Kenya	KEMRI-CDC	Kisumu	Regional	[1]ViroSeq	Plasma	227 *
				[1]In-house [2]	Plasma	120 *
					DBS	100 *
Senegal	ADUTH	Dakar	National	[1]ViroSeq	Plasma	155
				[1]In-house	Plasma	129
					DBS	155
Uganda	UVRI	Entebbe	Regional	[1]In-house	Plasma	200 *
					DBS	150 *
	JCRC	Kampala	National	[3]In-house [3]	Plasma/DBS	47.5
South Africa	NHLS-NICD	Johannesburg	Regional	[1]In-house [2]	Plasma	200 *
					DBS	225 *
	NHLS-WITS	Johannesburg	Regional affiliated	[1]ViroSeq	Plasma	460 *
				[1]In-house	Plasma	180 *
				[1]In-house	DBS	138 *
				[3]In-house [3]	Plasma/DBS	47.5
	ACVL	Durban	-	[4]In-house [4]	Plasma	100

Abbreviations: WHO, World Health Organization; DBS, dried blood spot
[1] Agence nationale de recherches sur le sida et les hépatites (ANRS) assay;
[2] CDC/ATCC/ThermoFisher assay;
[3] Affordable Resistance Test for Africa (ART-A) assay;
[4] Southern-African Treatment Resistance Network (SATuRN) assay;
* Cost inclusive of labor
Source: partly adapted from WHO 2013 HIV drug resistance reference laboratories prices. Available from: http://www.who.int/hiv/topics/drugresistance/HIVDR_genotype_pricelist.pdf?ua=1

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Chapter 15

The evolving landscape of HIV drug resistance diagnostics for expanding testing in resource-limited settings

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SUMMARY

Global scale-up of antiretroviral treatment has dramatically changed the prospects of HIV/AIDS disease, rendering life-long chronic care and treatment a reality for millions of HIV-infected patients. Affordable technologies to monitor antiretroviral treatment are needed to ensure long-term durability of limited available drug regimens. HIV drug resistance tests can complement existing strategies in optimizing clinical decision-making for patients with treatment failure, in addition to facilitating population-based surveillance of HIV drug resistance. This review assesses the current landscape of HIV drug resistance technologies and discusses the strengths and limitations of existing assays available for expanding testing in resource-limited settings. These include sequencing-based assays (Sanger sequencing assays and next-generation sequencing), point mutation assays, and genotype-free data-based prediction systems. Sanger assays are currently considered the gold standard genotyping technology, though only available at a limited number of resource-limited setting reference and regional laboratories, but high capital and test costs have limited their wide expansion. Point mutation assays present opportunities for simplified laboratory assays, but HIV genetic variability, extensive codon redundancy at or near the mutation target sites with limited multiplexing capability have restricted their utility. Next-generation sequencing, despite high costs, may have potential to reduce the testing cost significantly through multiplexing in high-throughput facilities, although the level of bioinformatics expertise required for data analysis is currently still complex and expensive and lacks standardization. Web-based genotype-free prediction systems may provide enhanced antiretroviral treatment decision-making without the need for laboratory testing, but require further clinical field evaluation and implementation scientific research in resource-limited settings.

INTRODUCTION

By the end of 2015, over 18.2 million people infected with HIV were receiving combination antiretroviral treatment (ART), reflecting an unprecedented scale-up of ART over the past decade[1]. This increased access to ART has been a major public health success especially in resource-limited settings (RLS) with significant reductions in HIV transmission and HIV-related mortality [2]. However, access to monitoring diagnostic laboratory tests has not matched the rapid ART scale-up [3–5]. Until recently, most programs in RLS have relied mainly on clinical and immunological criteria to monitor ART efficacy despite their inaccuracy and late detection of treatment failure when compared to the gold-standard viral-load criteria[3,6].

Following the recent World Health Organization (WHO) recommendations, implementation of routine viral-load monitoring is now being prioritized in large-scale ART programs to enable early detection of treatment failure, thus enhancing timely switching to next-line drug regimens[7,8]. In 2015, the Joint United Nations Programme on HIV/AIDS (UNAIDS) further launched an ambitious target for achieving 90% VL suppression rate among all patients on ART by 2020 [9]. This brings in focus the need for expanding access to HIV diagnostic tools.

With expansion of routine viral-load tests, the number of patients switched to second-line therapy is forecasted to increase to about 4-6 million by 2030, and this will comprise of nearly 20% of all patients on ART [10]. Limited available data have indicated that about 10-40% of the patients on protease-inhibitor-based second-line have treatment failure[11] and majority (70-90%) of them lack resistance mutations to the key drug, protease inhibitors (PI's) [12,13]. Due to the limited third-line drug options in most RLS, the clinical management of these patients is increasingly complex in the absence of HIVDR tests to determine those harboring drug resistant viruses and guide the selection of optimal regimens.

Rising levels of pre-treatment HIVDR in sub-Saharan Africa also put a threat to the success of national ART programs [14–16], especially in the era of 'treat all' and PrEP scale-up recommendations [17,18]. Recognizing these challenges, the recent WHO guidelines recommend active surveillance monitoring of HIVDR in patients initiating ART for pre-treatment HIVDR and in those on ART for acquired HIVDR [8,19].

Thus, to improve individual patient management, protect the durability of available drug regimens and ensure the rational use of scarce third-line drugs, there is a need for low-cost strategies to measure HIVDR. In this review we have assessed the landscape of current HIVDR tests and evaluated the strengths and limitations for use in both individual ART patient management and population-based surveillance and monitoring in RLS.

Landscape of HIV drug resistance technologies

HIV drug resistance tests are broadly classified into genotypic and phenotypic assays. Genotypic resistance tests (GRT) are sequencing-based assays that identify specific amino acid changes, or mutations, known to be associated with resistance to specific antiretroviral drugs. Genotypic resistance assays can be further categorized into sequencing based approaches such as; Sanger- and next generation sequencing and into assays based on the detection of specific point mutations. Although phenotypic assays may sometimes provide improved resistance assessments quality over genotypic tests [20], they are prohibitively expensive, require sophisticated laboratory set-ups such as biosafety level III facilities and have long turn-around times. Thereby, their use in resistance testing in RLS has not been realized and will not be discussed further in this review.

An ideal GRT for use in RLS has previously been described[5]. The specifications for such a test includes, low-cost, compatible with dried blood spots (DBS) specimen type, having broad subtype coverage, open access to use readily available consumables and reagents, and low cost instruments for sequencing/detection systems, as well as requiring minimal complexity in terms of equipment, analysis method and skills, and a reasonable turnaround time and acceptable testing sensitivity. It should also be able to pass certification and proficiency testing by appropriate regulatory authorities.

In the sections below we have discussed the current landscape of HIVDR tests first from a technical point of view and followed by an assessment of their applicability in RLS.

1 Sanger sequencing assays

Sanger sequencing, also known as first-generation sequencing assays, are based on di-deoxynucleotide chain-termination technique [21] and are currently the most widely used assays. Sanger sequencing requires four basic steps: nucleic

acid extraction, target amplification, sequencing and data analysis (Figure 1). Briefly, extracted nucleic acid from either plasma or DBS is first amplified using primers for the target region. The confirmed amplicons are then sequenced using fluorescently labeled chain terminators to generate nested sets of dye-labeled products, which are then resolved by gel or capillary electrophoresis in a genetic analyzer. The sequence data are then edited and consensus sequences generated analyzed for HIVDR using proprietary software or web-based HIVDR databases that provide automated sequence interpretations [22].

Sanger GRT includes commercialized kit-based and in-house assays (**Supplementary tables 1 and 2**) [23–32] . To date, only two genotypic assays for the *polymerase* gene (protease and reverse-transcriptase) have been FDA-approved i.e. ViroSeq assay (Celera Alameda, CA USA) and TruGene assay (Siemens Healthcare Diagnostics, Deerfield, IL USA), with the latter having been discontinued [26]. ViroSeq covers the entire protease region (1-99 codons) and two-thirds (1-335 codons) of the reverse-transcriptase region [23]. It has a detection limit of 2,000 cps/ml of plasma viral load and is approved dedicated for use with certain models of the Applied Biosystems genetic analyzers and for HIV-1 subtype B viruses only.

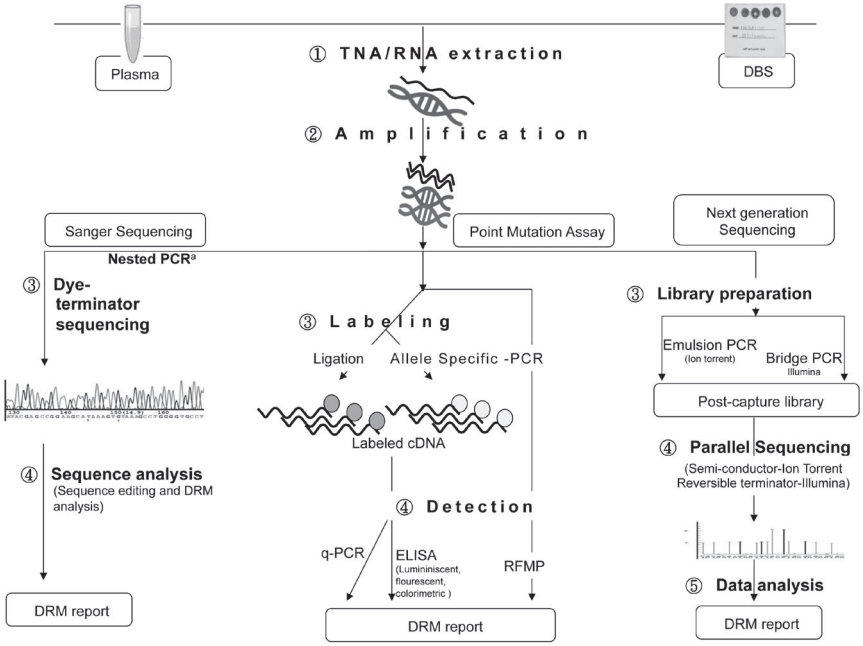

Figure 1. Overview of Sanger, point mutation, and next generation assays work flow.

(1) Nucleic acid is extracted from plasma, RNA, or dried blood spots; TNA, followed by (2) amplification to either cDNA or DNA. *Some assays include a second amplification to increase yield. For Sanger sequencing (3) dye-terminator sequencing involving incorporation of fluorescence-labeled terminator bases to produce dye-labeled nested products, which are resolved in a genetic analyzer to produce a chromatogram. (4) Analysis involves base-calling, sequence editing, and quality checks by use of automated software followed by drug resistance analysis using proprietary or web-based applications. For point-mutation assays, (3) production of labeled target product is done where labeled bases are incorporated in complementary DNA strands using either selective ligation of adjacent oligonucleotide probes by a template-dependent DNA ligase or single base extension by allele-specific primers. (4) Detection of labeled target mutation then occurs by q-PCR or ELISA. Alternatively non-labeled products of different lengths are generated using allele-specific primers of varying lengths.
The products are then digested using restriction enzymes and detected through mass-spectrometry. For next-generation sequencing, (3) a library of target nucleic acid is created from the initially amplified DNA by a second round of amplification in a process called target enrichment. First, the DNA or cDNA is randomly fragmented to form short strands that are then joined to adapter sequences template (adaptors contain binding sequences, barcodes and primers). The se then undergo amplification, either by emulsion PCR (Ion Torrent) or bridge PCR (Illumina). (4) Sequencing is by synthesis through semi-conductor sequencing – Ion Torrent or reversible termination sequencing: Illumina (5). Analysis involves a series of steps involving (a) quality assessment of the raw files, (b) removal of adaptor sequences, (c)de-multiplexing to re-identify the samples, (d) mapping of data to reference sequence variant calling and validation, and (e) HIVDR analysis by third-party resistance interpretation databases. cDNA: complementary DNA; DBS: dried blood spots; DRM: drug resistance monitoring; HIVDR: HIV drug resistance; RFMP: restricted fragment mass polymorphism; TNA: total nucleic acid.

Potential for use in RLS

Many research or public health laboratories around the globe have developed in-house Sanger assays for HIV GRT, to circumvent the high costs associated with the commercial tests and to ensure compatibility with HIV-1 non-B subtypes

[24,25,27,29–32]. The following three laboratory developed and validated in-house assays have been commonly used in sub-Saharan Africa : 1). The assay that was developed by the French National Agency for AIDS Research (ANRS) [30], 2). The assay that was developed by the US Centers for Disease Control and Prevention (CDC) and the technology had been transferred to the American Type Culture Collection (ATCC) and Thermo Fisher [31,33] scientific for kit productions 3). The Southern African Treatment and Resistance Network (SATuRN) developed assay which is partnering with Thermo Fisher for kit-based production [29,34]. There are other laboratory developed in-house assays, such as the RT only Affordable Resistance Test for Africa (ARTA) assay [32] and low-cost assays from Asia-Pacific region [24,25,27] (See supplementary table 2 for details).

Compared to other potential GRT, these Sanger-based sequencing assays are the ones commonly used for WHO- recommended HIVDR surveys [35]. These assays have demonstrated optimal performance over a wide range of HIV-1 subtypes and circulating recombinant forms (CRFs) and some of them had performed well on DBS sample type with high accuracy and reasonable (1,000-5,000 cps/ml of viral load) genotyping sensitivity [30–32,36] and have a reasonable cost per test (USD 40 to 100), (Supplementary table 2). Due to the relatively low-cost per test and medium to high throughput of the genetic analyzers used, they are suitable for both individual-patient monitoring and HIVDR surveys.

In addition, a number of open-source sequence editing tools are available for use with Sanger-based assays including automated tools that minimize inter-subject variability. This includes Recall (University of British Columbia, Canada) and Bioedit (Tom Hall, CA USA).

However, there are limitations for Sanger-based assays: They are labor-intensive, require certain laboratory set-ups for preventing contamination and only detect majority genotypes (>20%). Moreover, they use sequencers that have a high capital cost (~250,000 USD) whose infrastructure is mainly suitable for centralized specialized laboratories.

2 Next-generation sequencing assays

Recent advancements have seen the development of massive parallel high-throughput sequencing technologies collectively called next-generation sequencing (NGS) [37]. Although NGS technology has rapidly transformed the landscape of most areas in genomic research [38], its introduction into routine

clinical application has been much slower. Today only a few commercial virus genotyping assays based on NGS platforms are available; DeepGen Assay (University Hospital Case Medical Center) using IonTorrent and Monogram's GeneSure Genotypic Assay using Illumina's MiSeq sequencers [39]. The Illumina Miseq and IonTorrent NGS are the most commonly used platforms in HIVDR research and surveillance [40] and are also the potential low-cost bench-top assays discussed in this section (Supplementary table 3).

The basic workflow for NGS include nucleic acid extraction, amplification, library preparation, target enrichment, sequencing, imaging and data analysis as shown in Figure 1.

Potential for use in RLS

Similar to Sanger-based sequencing assays, NGS also have a relatively high capital cost for acquiring instruments, but this is expected to gradually decrease with advancements that allow competition and innovation of even cheaper technologies. Currently, the only commercially available "low-cost" devices are the bench-top Ion Torrent PGM (USD 50,000), Illumina's Miseq (USD 99,000) and MiniSeq (USD 49,500) [41].The cost per run of NGS is prohibitively high for use in RLS (Supplementary Table 1), but the prices can be significantly lowered through multiplexing [42,43]. A previous study using Illumina Miseq (read length ~200bp) demonstrated that 24 samples could be multiplexed with a depth of >10,000 counts per base [43] at a cost of between 24-31 USD. An even higher level of multiplexing has been demonstrated in 'wide-sequencing' approach (Multiplexing of many samples in a run but with lesser coverage depth) on Illumina Miseq resulting with sequencing costs of ~5 USD per sample (multiplex of 1,143 samples) with a median read depth of >9900 counts per base [44]. This study however only sequenced a small portion of RT (90-234 bases) but the same depth and costs may not be achieved when longer-targets are desired.

Sequencing of longer-genome targets is sometimes required especially when assessing resistance in patients treated with intergrase-strand-transfer inhibitors (INSTI) plus nucleoside reverse transcriptase inhibitors (NRTIs) or PI's without the need for separate assays as is the current case for most available Sanger-sequencing assays [39]. This is especially important, given the current recommendation for use of INSTI with NRTIs or/and PIs combinations in RLS. As with Sanger-based assays NGS also have the ability to use the widely validated broad subtype primers and DBS [45], as they share similar steps in the upstream procedures (Figure 1).

Limitations for NGS include the following: The need for high-level multiplexing to achieve significant cost-reductions limits their use to high throughput facilities or for population-based HIVDR surveys. In addition the assay requires multiple procedures, which increases the complexity of the assay and the laboratory infrastructure. Moreover the cost of library preparation for longer-genome targets is expensive and does not change even with increased order of multiplexing (Figure 2).

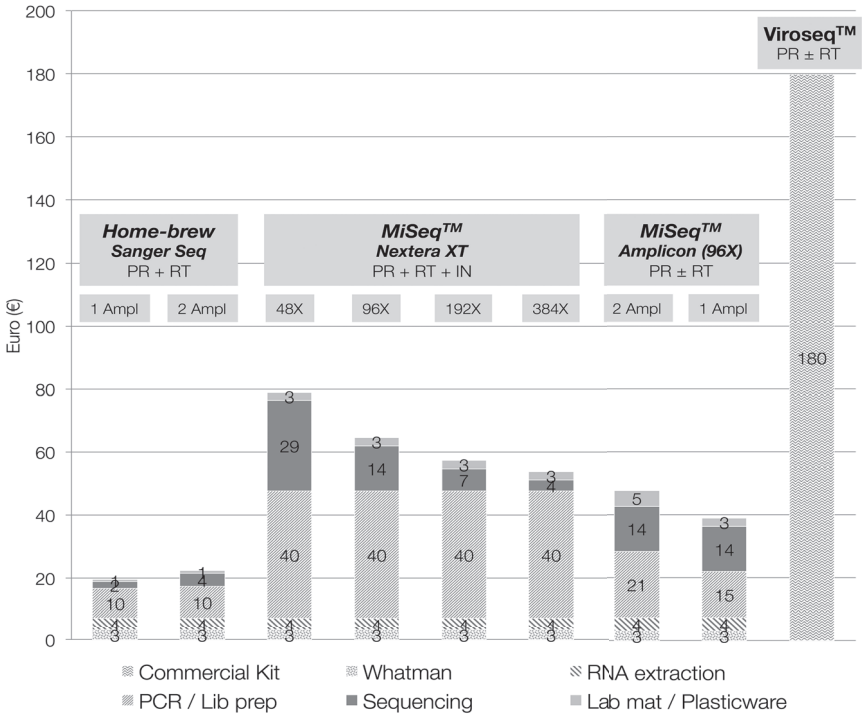

Figure 2. Reagent costs of HIV-1 genotyping using different technical approaches in a European laboratory.

These are real reagent costs in a specific European laboratory in 2015 and may vary from lab to lab. They do not include workforce costs, upfront spending on equipment acquisition, or maintenance. This comparison show that both homebrew Sanger sequencing and next generation sequencing can reduce sequencing costs by at least twofold compared with current standards. A MiSeq™ Nextera XT approach would provide resistance data for protease, reverse transcriptase, and integrase, being optimal for current genotyping needs. However, such an approach remains significantly more expensive than homebrew Sanger sequencing. Of note, the current main driver of sequencing costs for the MiSeq™ Nextera XT approach is library preparation. Whereas sample multiplexing might reduce sequencing costs to some extent, truly significant cost reductions must come from reducing library preparation costs. Lab mat: laboratory materials; Lib prep: library preparation; IN: integrase; PR: protease; RT: reverse transcriptase; Seq: sequencing; 96X: 96 reactions.

The level of bioinformatics support, expertise and infrastructure required for on-site data analysis is also still complex and expensive [46]. This includes the need

for dedicated data center with servers and storage center, computing clusters (high performance computers, high capacity storage and fast networks) and skilled bioinformaticians, system administrators and developers [46]. Alternative cloud based analysis applications are also expensive, for example the DeepChek (Advanced Biologic Laboratories, Luxembourg). This is currently the only commercially available HIVDR customized bioinformatics application, developed for research use only, and has a cost of USD 65 per sample (~3 times the cost of sequencing) [47]. However, there are ongoing efforts to create open-source robust yet user-friendly automated NGS bioinformatics HIVDR analysis platforms that could be used by laboratory technicians with no bioinformatics expertise.

3 Point Mutation Assays

Point mutation assays identify only specific mutations as opposed to the sequencing methods which provide sequence information of almost the entire target genome. A number of point mutation assays have been developed and adapted for use in HIVDR [48–60]. These include allele specific PCR (ASPCR) [48,51–56,58–60] and oligonucleotide ligation assays [49,50,57]. ASPCR assays use mutation specific primers with mismatched 3′ residues to selectively amplify viruses containing mutant and wild-type alleles of a given codon. Ligation assays on the other hand use the principle of selective ligation of adjacent oligonucleotide probes by a template dependent DNA ligase [49,50,57]. The oligonucleotides are designed to selectively match the mutation site while mispriming if hybridized to a wild-type template. The target allele is then detected by either ELISA (colorimetric, fluorescent or luminescent) [48–50,53,58,59] real-time q-PCR [54,55,57,60], mass spectrometry [52] or gel electrophoresis [56] (Figure 1).

Potential for use in RLS

Compared to the other promising low cost technologies, point mutation assays are an attractive option as they have a lower capital cost and can also be deployed at or near point-of-care settings. A key challenge is that they are only able to identify a limited number of mutations. Furthermore, a high polymorphism at primer sites affects both the accuracy and sensitivity of these tests. Equally high codon redundancy at mutation site coupled with the high number of possible resistance mutations for the different drugs in the combined regimen increases the cost per sample in the absence of high order multiplexing. Moreover differential codon usage by HIV strains makes point mutation assays subtype dependent.

Based on a recent analysis of genotypes available in the Stanford HIVDR database, it was proposed that a point mutation assay capable of detecting a set of six drug resistance mutations in reverse-transcriptase (i.e. K103N, V106M, Y181C and G190A [NNRTIs] and K65R and M184V [NRTIs]) could be sufficient to detect drug resistance in patients failing first-line and in antiretroviral-naïve patients, with 98.8% and 61.2% sensitivity, respectively [61]. A similar analysis using the same data indicated that there are up to 42 distinct codons for the proposed set of six DRMs in sequences from seven most common subtypes (A, B, C, D, G, CRF01_AE, and CRF02_AG) [62]. This ranged from four codons at the 184 position, to eleven at position 190.

A proposed solution to use degenerate primers (a mix of primer sequences with some positions having a number of different possible bases) is feasible [63,64] to cater for polymorphisms near the target binding sites but this may affect specificity and will still require a medium-high level multiplexing capability due to high codon redundancy at target sites.

Most point mutation assays described in literature, have a low multiplex ability due to limitations in available technology and instrumentation [49–51,54,55,57,58]. Some assays are able to compensate for this by using high throughput instruments but at an increased labor, complexity and cost which makes it difficult to deploy them at point of care. Only the array based assays show promise with medium to high-level multiplex ability, but they still rely on molecular systems that are difficult to deploy at point of care [48,52,59]. Examples include the micro-array-based multiplex-allele specific drug resistance assay (MAS-DR) [59] and the MALDI-TOF mass-array assay [52]. Of these assays, only the MAS-DR assay has both a high multiplex ability and a low-capital cost. A study by Zhang et al using MAS-DR multiplexed 45 allele targets (20 wild type and 25 mutants) [59], at a cost of 40.90 USD using a low cost Luminex Magpix (~27,500 USD) device. The mass-array assay has also been shown to have a low cost, at USD 30 for 18 target alleles but the mass-spectrometers are costly ranging from USD 150,000 to 850,000 (Supplementary table 4).

4 Genotype-independent predictions systems of treatment response

The exponential growth in the collection of biomedical data has the potential for the development of personalized treatment decision-making. An example of where "Big Data" or artificial intelligence could inform individualized medicine is that of HIV treatment. These so-called genotype-free systems for predictions of ART responses provide a practical and affordable alternative to laboratory-based strategies to enhance ART decision-making[22,65]. Typically, the data used to train the underlying computational models to make predictions include a complete ART history, VL and CD4 count on the failing ART regimen. The most advanced initiative to date is the HIV Treatment Response Prediction System (HIV-TRePS) developed by HIV Resistance Response Database Initiative's (RDI) and it is available as a free web-service[65]. Using biological, clinical and treatment outcome data from more than 180,000 HIV-1 patients derived globally, RDI has developed computational models that can accurately predict HIV treatment outcomes, which may be used to identify optimal and individualized therapies for patients experiencing ART failure[65]. The latest models have achieved an accuracy of predicting ART response at the level of 82%, which is statistically significantly more accurate than GRT's rules-based interpretation, typically at 55-65%[65].

Potential use in RLS

It seems that the non-genotype prediction systems are the most cost-effective method in drug resistance apart from the requirement for Internet infrastructure. Studies also showed that the methods resulted in more accurate prediction of treatment outcomes than the traditional genotype-dependent rule-based algorithms incorporated in most HIVDR interpretation databases [22,65]. The accuracy is however lower for regions in which there are limited datasets in the database, such as for sub-Saharan Africa[65].

In addition, the prerequisite of the model systems for a recent VL and CD4 test result[65] may have limited it's utility in RLS as most countries have adopted the 2015 WHO-recommendation for 'test and start' treatment strategy and viral-load based monitoring leading to reductions in CD4 testing [8]. In addition, not all patients having virological failures have clinically relevant resistance mutations, thus these methods might result in unnecessarily switching to costlier regimen, potentially making them more expensive than GRT. Lastly, these systems also have limitations in providing data at the population level to guide programmatic decisions and hence may only be useful for individual patient management.

Expanding testing within the current health infrastructure

At present HIVDR testing in Africa, is limited to only a few laboratories, mainly research facilities and national reference laboratories, some of which are WHO-designated national or regional HIVDR laboratories[5]. Expansion of drug resistance testing to new sites may be hampered by the high capital costs, limited infrastructure, quality assurance requirements, and shortage of high skilled and experienced personnel. Under the current landscape, incorporation of HIVDR testing in the public-health system could best follow a centralized approach within the WHO recommended tiered framework for health-care delivery [66]. This tiered approach incorporates an integral laboratory system aligned within the country's public health delivery structure of the four-level hierarchal health system.

The WHO-designated national/regional HIVDR laboratories belong to the top tier of the four level pyramid system [66]. These laboratories serve as HIVDR referral facilities for in-country peripheral sites and nearby countries where genotyping capacity is lacking. Appropriate technologies for use in these laboratories would include the Sanger-sequencing based assays, multiplex point mutation assays and NGS. These could mainly be the high throughput facilities, which may in addition have bioinformatics capacity to support NGS analysis.

For l*evel III facilities (*provincial/ regional laboratories within a country), these can also incorporate upcoming point mutation assays such as the Luminex Mapgpix. Level III facilities can also perform PCR tests and then send the products to level IV laboratories for sequencing as these laboratories have the capacities for performing molecular tests, such as viral-load test and DNA-PCR for early infant diagnosis. Moreover, facilities at level III could also serve as sample collection sites to the referral centers.

As with the WHO recommendations, the level II (district) and level I (primary health care centers) laboratories could serve as sample collection sites for referrals to the level III and IV facilities. In addition to the tiered approach, the genotype-free prediction systems could be incorporated directly at the clinician's office to support decision-making in selecting most effective treatment regimen if the prerequisite for a recent VL and CD4 test results are available at the sites for patients.

Quality Assurance

The expansion of HIVDR testing will also require the strengthening of quality management systems to ensure accurate, timely and reproducible results

reporting. Consideration should be given to the entire quality management cycle: quality-assured sample collection and timely sample transportation (pre-analytic); standardized and valid testing procedures (analytic), and systematically reviewing and timely reporting results process (post-analytic). This requires the use of standard operation procedures (SOPs), sample and results tracking devices coupled with continuous training and supervision [5,67]. In addition, laboratories also need to implement sequence quality assurance systems to ensure and monitor consistency in assay performance quality, which includes sequence editing, assess for contamination and other sequence quality aspects such as sequence length, stop codons, unexpected insertions and unusual residues.

Testing facilities will need to be accredited with appropriate standards such as ISO-15189 for medical laboratories, in addition to obtaining WHO designation for facilities within WHO-HIVDR laboratory network. Facilities also need to participate in routine external quality assurance (EQA) schemes for proficiency testing. Laboratories can leverage on existing regional agencies to facilitate both accreditation and EQA schemes, as is the case of TREAT Asia quality assessment scheme (TAQAS) [68] and WHO-mediated step-wise laboratory improvement towards accreditation (SLIPTA) scheme [69].

DISCUSSION

ART management in RLS continues to be challenging due to emergence of HIVDR and limited available drug options. As with high-income countries the use of GRT can play a vital role in managing patients with suspected treatment failure [5,8,70]. As viral load monitoring for ART patients become a routine care and treatment package in RLS[7], drug resistance testing for patients with confirmed virologic failures will become a reality. In fact, several PEPFAR-supported countries in sub-Sahara Africa are recommending drug resistance testing for patients with 2nd-line treatment failures (South-Africa and Kenya national treatment guidelines) [71,72]. This is in addition to the WHO recommended population-based surveys; pre-treatment HIV drug resistance, acquired HIV drug resistance (12 months and ≥48 months) as well as surveillance of HIV drug resistance in children<18 months of age [19]. These routine surveys are vital in guiding the choice of first-line treatment, pre and post-exposure prophylaxis prevention regimens and subsequent second- and third-line regimens[19].

Within the current technology landscape, expansion of HIVDR testing in RLS, may depend on low cost Sanger-sequencing based in-house assays, low cost bench-top NGS, and possibly point mutation assays with medium to high level multiplex capability. Each of these technologies has their strength and limitations. While Sanger-based in-house assays are the most commonly available, capital cost for the sequencers and per test cost is comparatively high and this may limit their use, especially in sites considering starting GRT. On the other hand, they have been widely validated and there is considerable expertise for their use in RLS. Moreover, some of the assays, such as the CDC/ATCC/Thermo Fisher Scientific assay, are already commercially available as a testing kit or being developed (SATuRN collaboration with Thermo Fisher Scientific) into kit-based assays, which is not the case with the other low-cost technologies.

Next-generation sequencing has an equally high capital cost but it is projected that this may gradually decline as more novel technologies become available. Though the cost per run for the NGS is high, significant cost reduction can be achieved through multiplexing of many individually barcoded samples. On the other hand, the need for multiplexing implies that its utility is limited to high-throughput facilities. Moreover, they also have a high analysis cost with cloud-based computing tools being up to three times higher that of the actual lab test. A number of open source analysis pipeline are available but the level of expertise and computing infrastructure may still be limiting for most RLS. Different academic laboratories are however undertaking efforts to simplify the analysis process and minimize the analysis costs.

While point mutation assays might seem the likely choice for point of care testing, they are highly limited by the multiplexing ability of the technique used. Although there are various variants of point mutation assays, optimization of these assays to accommodate HIV variability and polymorphisms as well as adapting these assays for use with low-cost high multiplex instruments, could be difficult to implement in the short-term. Further basic and technological research coupled with engineering advances is needed to make point of care assays truly feasible.

Lastly the genotype-free prediction systems could easily be adapted with limited capital cost in RLS to improve the management of patients with suspected treatment failure. A setback of this method is the potential for switching patients

without resistance to the expensive next-line of treatment and the need for CD4 results, which may not be available under the current strategy for test and start and viral-load based monitoring. In general, all these technologies may require additional field evaluation to assess their suitability in given settings as well as their cost-effectiveness.

To date, implementing HIVDR testing under the current landscape could best follow a centralized approach embedded in the recommended WHO tiered approach with testing at the national or regional centers. The lower facilities could then serve as sample collection sites supported by sample referral and data management systems to ensure quality of sample collection, timely shipment and results reporting. In addition, the level III laboratory tiers with molecular-based systems for viral load and DNA-PCR for early infant diagnosis can also incorporate low-cost point mutation assays like the luminex magpix assay or serve as PCR amplification laboratories for DR testing. As an alternative to laboratory-based tests, genotype-free prediction systems can also be used directly at the clinician's office, although these predictions are based on indirect parameters, i.e VL and CD4, rather than the direct detection of resistance mutations.

In conclusion, the current landscape of HIV drug resistance technologies shows promise with low-cost assays that can be used to expand testing for both clinical management and surveillance in sub-Saharan Africa. However more implementation research is urgently needed to operationalize the use of these technologies within the public health system in RLS. This type of research should be planned in light of the ongoing global expansion of HIV viral load testing in RLS.

Contributors

SCI, RLH and TFRW wrote the first draft of the paper. All authors contributed to subsequent versions, and have read and approved the final paper.

Conflicts of interest

We declare that we have no conflicts of interest.

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Supplementary table 1: Examples of Sanger-based HIV drug resistance tests in LMIC

a) Viroseq™ HIV-1 genotypic assay

Company/Institution	***Abbott**
Product Name	Viroseq
Core Technology	Di-deoxynucelotide sequencing using ABI Genetic analyzer series
Technology set-up	Not-fully automated, pre-sequencing manual procedures, semi-closed system
Target region	HIV-1 protease gene from codons 1-99 and two-thirds of the reverse transcriptase gene, from codons 1-335
HIV-1 subtypes	FDA approved for subtype B, varied performance with non-B sub-types
Sample types	Mainly Plasma, but can be performed in DBS and PBMCs
Sample volume	500µl
Sample preparation/RNA extraction	ViroSeq
Assay description	One step RT-PCR assay with 6 sequencing primers
Sequencing	Sanger method, big dye terminator chemistry, ABI prism capillary based detection
Amplification sensitivity	>2000 cps/ml but can go as low as 500 cps/ml
Time to results	~17-32 hrs
Core equipment	ABI genetic analyzer series
*Cost/test	$155-380
Technical skills	High
HIVDR interpretation	ViroSeqTM HIV-1 Genotyping System's dedicated software
Laboratory set-up	Level 2 laboratories, 2-4 dedicated rooms
Regulatory approval	FDA approval, commercially available
Strength	FDA approval, commercially available, standardized, in-built system for contamination, and system for sequence analysis
Weakness	High cost/test, high infrastructure cost, need for high technical skills, sub-optimal performance on some subtypes and DBS
Availability	Widely available through ABI/ThermoFisher Scientifics

***Excluding labor (http://www.who.int/hiv/topics/drugresistance/HIVDR_genotype_pricelist.pdf?ua=1)

b) Examples of published in-house genotypic assays commonly in use in sub-Saharan Africa

Company/Institution	ANRS [1]	CDC/ATCC/Thermo Fisher [2,3]	SATuRN [4,5]
Product Name	N/A	ATCC	N/A
Target region	Separate PR (507bp) and RT (798bp-20-240)	HIV-1 protease gene from codons 13-99 and 1-250 of reverse transcriptase (RT) gene	HIV-1 protease gene from codons 1-99 and 1-300 of reverse transcriptase (RT) gene
HIV-1 subtypes	Major group M viruses, A, B, C, D F and G and CRF01-AE and CRF02-AG	Major group M viruses, A, B, C, D F and G and CRF01-AE and CRF02-AG	Major group M viruses, A, B, C
Sample types	Plasma, DBS	Mainly plasma	Plasma
Sample volume	1ml plasma, 2 spots for DBS	140µl plasma, 1 DBS spots (50µl)	200µl plasma
Extraction method/sample preparation	Nuclisense/Abbott/ QIASymphony	Nuclisense/Qiagen/Abbott	Qiagen
Assay description	Separate PR, and RT	RT-PCR, Nested with 6 sequencing primers	RT-PCR, Nested with 4 sequencing primers
Amplification sensitivity	5000cps/ml DBS (60% of amplification for VL comprised between 51 and 200 cps/ml)	500cps/ml for plasma and 1000cps/ml for DBS	1000cps/ml plasma
Time to results	4 days	16-24 hours	-
Core equipment	ABI genetic analyzer series	ABI genetic analyzer series	ABI genetic analyzer series, Beckman Coulter CEQ
Cost/test	-	$50 for WHO-HIVResNet and PEPFAR supported laboratories	$100, 25% reduction for South African resistance network members
Technical skills required	High	High	High
Laboratory set-up	Level 2 laboratories, 2-4 dedicated rooms	Level 2 laboratories, 2-4 dedicated rooms	Level 2 laboratories with 2-4 dedicated rooms
Regulatory approval	None	None	_
Strength	Low cost/test, widely used in west & central sub-Saharan Africa, use of DBS, sample extraction using existing platforms for other tests	Low cost/test, kit-based commercially available, widely evaluated & in use in many settings, sample extraction using exiting platforms for other tests, optimum performance with DBS and across group M subtypes	Low cost/test, heading to kit-based
Weakness	High instrument and maintenance cost, need for high technical skills, not commercially available	High instrument and maintenance cost, need for high technical skills	High instrument and maintenance cost, need for high technical skills
Availability	Available in most ANRS affiliated laboratories	Available as a kit sold by ThermoFisher Scientifics	To be made in a kit through collaboration with ThermoFisher Scientifics

Sequencing is either by ABI or Beckman series genetic analyzers. HIV drug resistance interpretation is by any of the online databases, which include Rega, ANRS, HIV-GRADE, Euresist, HIV-TRePS. ANRS: National agency for AIDS research; CDC/ATCC: Centers for disease control and prevention/ American Type Culture Collection; DBS: dried blood spot; HIVDR: HIV drug resistance; PBMC:peripheral blood mononuclear cell; PR:protease; RT:reverse transcriptase; SATuRN: Southern African treatment and resistance network; VL:viral load

c) Examples of published in-house genotypic assays in Asia-Pacific region

Company/Institution	**National AIDS research institute India[6]**	**Hong Kong university[7]**
Product Name	N/A	N/A
Target region	1204 bp of both PR and RT genes	HIV-1 protease gene from codons 1-99 and 1-400 of reverse transcriptase (RT) gene
HIV-1 subtypes	Major group M viruses, A, B, C	Major group M viruses A B C D G & CRF_001 AE, CRF_002AG,CRF07_BC, CRF08_BC, CRF33_01B
Sample types	Plasma	Plasma
Sample volume	500µl plasma	500µl plasma
Extraction method/sample preparation	Qiagen	Qiagen
Assay description	RT-PCR, Nested with 6 sequencing primers	RT-PCR, with 7 sequencing primers
Amplification sensitivity	1000cps/ml	400cps/ml
Time to results	16 hours	17-32 hours
Core equipment	ABI genetic analyzer series, Beckman Coulter CEQ,	ABI genetic analyzers
Cost/test	$112	$40
Technical skills	High	High
Laboratory set-up	Level 2 laboratories with 2-4 dedicated rooms	Level 2 laboratories with 2-4 dedicated rooms
Regulatory approval	_	_
Strength	Low cost/test	Low cost/test
Weakness	High instrument and maintenance cost, need for high technical skills	High instrument and maintenance cost, need for high technical skills, not commercially available
Availability	Localized	Localized

Sequencing is either by ABI or Beckman series genetic analyzers. HIV drug resistance interpretation is by any of the online databases, which include Rega, ANRS, HIV-GRADE, Euresist, HIV-TRePS

Supplementary table 2: Commonly used next-generation sequencers

Company	Illumina Bioscience [8]	Illumina Bioscience[8]	ThermoFisher scientifics[8]
System	Illumina (MiniSeq)	Illumina (MiSeq)	Ion Torrent PGM 318 chip
Amplification Method	Bridge PCR	Bridge PCR	Emulsion PCR
Principle (Chemistry)	Synthesis (reversible termination)	Synthesis (reversible termination)	Synthesis (H+ detection)
Instrument Cost (USD)	$49.5K	$99K	$50K
Equipment supplied with core system	Single unit inclusive of amplification, sequencing, paired-end, and integrated analysis	Single unit inclusive of amplification, sequencing, paired-end, and analysis hardware	Dell Precision T7500 Server
Reagent Cost (USD) (library + run)	$729	$725	$749
Max Read Length (bp)	~300	~300	~ 200
Max Yield / run (Gb)	~7.5	~15	1
*Cost/sample (USD)			
Maximum No. of libraries per run	384	384	384
Total sample prep time	~6 hrs	~2 hrs	~2 hrs
Sequencing time excluding sample prep	24 hrs	39 hrs	2 hrs
Primary Error (Error Rate)	Substitution >0.1%	Substitution >0.1%	Indel in homopolymeric regions and CAFIE errors~1%
Primary Advantage(s)	Easy workflow, Fast run	Easy workflow, Fast run	Low cost, Fast run, potential for longer read
Primary Disadvantage	Short reads complicate haplotype reconstruction (likely not clinically relevant)	Short reads complicate haplotype reconstruction (likely not clinically relevant)	Homopolymer misreads
Availability	Through Illumina Bioscience, also allows a trade-in with other NGS like 454 & Solid and ABI series 3100 through 3730xl	Through Illumina Bioscience, also allows a trade-in with other NGS like 454 & Solid and ABI series 3100 through 3730xl	Through ThermoFisher Scientifics, allows a trade-in with ABI 3130xl

***** Depends on the level of multiplexing and the type of library preparation used but can go as low as <1 USD
CAFIE: Carry forward, incomplete extension; Indel: insertions deletions errors; errors; PCR: polymerase chain reaction.

Supplementary table 3: Published point mutations assays

Product Name	Oligonucleotide ligation assay [9,10]	Heteroduplex mobility assay[11]	Ligation-Amplification assay[12]	Parallel-Allele specific PCR assay[13]	Multiplex allele specific-drug resistance assay[14]
Company/ academic institution	University of Washington	Johns Hopkins University	Duke University Medical Center	CDC	
Principle	Template-dependent ligation of 2 primers and detection by ELISA	Selective hybridization to labeled probes with insertions or deletions at target mutation sites to form heteroduplexes, detection by gel-electrophoresis	Template-dependent ligation of 2 primers and detection either by ELISA or quantification with q-PCR	Single-base allele sequencing of polonies fixed to an acrylamide surface using labeled nucleotides, detection with micro-array scanner	Selective primer extension by biotin labeled dNTP's, hybridization to solid microspheres and detection by use of luminex micro-array system
Core equipment	Plate spectrophotometer	Gel electrophoretic equipment, radio imaging	q-PCR equipment	Microarray scanner	Luminex suspension micro-array system
Sensitivity	<1–12	0.03-0.05%	<1–12	0.003 - 0.4%	0.015-0.125%
No of mutations	1 per reaction	1 per reaction	1 per reaction	Multiple, but 1 in every sequence round	Up to 20 in a multiplex reaction
Linked mutations	No	Yes	No	Yes	Yes
Labor intensity	Minimal	Minimal	Minimal	Intensive	Moderate
HIV-1 Subtypes	Mainly optimized for subtype B but evaluated and modified for other group M subtypes	Subtype B	Subtype A, B C and D	Subtype C	Subtypes A, B, C, D, F, G, H, CRF01_AEs, CRF04_cpx
Time To Result	6-8 hrs	-	-	-	~12 hours
Cost	$1.50-$2.5 per assay (2013 price)	-	-	-	$ 40.90 (U.S. market price 2013)
Main Instrument cost	-	-	-	<$10k	~$27.5k
Strengths	Sensitive, rapid and affordable	Affordable, multiplex, sensitive	Sensitive, affordable	Sensitive, enables linkage of mutations	Sensitive, multiplex capability of up to 20 mutations, affordable
Weaknesses	Only 1 to 3 allele per reaction, specificity, affected by polymorphisms, low amplification sensitivity in non-B subtypes	Potentially low specificity for close mutations and polymorphisms at neighboring sites, no wide validation done	Only 1 allele per reaction, Specificity affected by polymorphisms	Relatively high cost, Labor intensity	Alelle primers subtype dependent, no wide validation done

dNTP: deoxynucleotide triphosphate; qPCR: quantitative polymerase chain reaction.

Supplementary table 3: Published point mutations assays (cont)

Product Name	Mutagenic specific PCR assay[15]	Matrix-assisted laser desorption and ionization time of flight mass spectrometry assay [16]	Micro-array mini-sequence[17]	Real-time Allele specific PCR[18–20]	Enzyme-linked minisequence assay [21]
Company/ academic institution		GeneMatrix Inc S.Korea			AIDS Research Center, NIID,Japan
Core technology	Gel electrophoresis	Restriction fragment mass polymorphism	Micro-array PCR	q-PCR	ELISA
Equipment	Gel electrophoretic equipment	MALDI-TOF MS	Micro-array scanner	q-PCR	ELISA readers
Principle	Competitive amplification by use of Wt and Mutant primers of different lengths and detection via gel-electrophoresis	Allele specific extension and mass measurement of the mutation containing amplicons	Single-base extension by labeled dNTP's, hybridization to solid sphere and detection by micro-array scanner	Differential amplification of mutants vs. WT in real-time PCR	Single-base allele sequencing using biotin-labeled dNTP's and detection by ELISA
Sensitivity	0.10%	0.05%	-	0.10%	0.10%
No of mutations	1 to 2 per reaction	18 but possible to assess all target mutations	25 but possible to assess all target mutations	1 per reaction,	1 per reaction
Linked mutations	Yes	Yes	Yes	Yes	No
Labor intensity	Minimal	Moderate	Intensive	Moderate	Moderate
HIV-1 Subtypes	A, B, C, D, E, F G and AE, but no wide validation done	Mainly optimized for subtype A, B C and D	Assessed on subtype A, C, D and CRF_01AE	Mainly optimized for subtype B and C	No validation done on subtypes
Time To Result		~7-8hrs			
Cost	$12.50	$30	-	$10-15	-
Main Instrument cost	-		<10k$		-
Strengths	Affordable, sensitive, relatively higher specificity	Affordable, sensitive	Potentially affordable	Sensitive, affordable	Affordable, sensitive
Weaknesses	Allele specific primers are subtype dependent and influenced by polymorphism at target site, no wide validation done	Complex equipment required, dependent on known information on molecular mass of mutants which may vary with polymorphisms and subtypes, not widely validated	Labor intensive, Allele specific primers influenced by polymorphisms and subtypes, not widely validated	Specificity affected by polymorphisms, multiple variants exists, not standardized and not widely validated	Allele may be subtype dependent and influenced by polymorphisms, no wide validation done
Availability	Not commercialized	Not commercialized	Not commercialized	Not commercialized	Not commercialized

dNTP: deoxynucleotide triphosphate; MALDI-TOF: matrix assisted laser desorption ionization-time of flight mass spectrometry; qPCR: quantitative polymerase chain reaction.

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Chapter 16

Clinically-relevant thresholds for ultrasensitive HIV drug resistance testing: a multi-country nested case-control study

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SUMMARY

Background

Implementation of ultrasensitive HIV drug resistance tests for routine clinical use is hampered by uncertainty about the clinical relevance of drug-resistant minority variants. We assessed different detection thresholds for pretreatment drug resistance to predict an increased risk of virological failure.

Methods

We did a case-control study nested within a prospective multicountry cohort. Our study included patients from 12 clinical sites in Kenya, Nigeria, South Africa, Uganda, and Zambia. We defined cases as patients with virological failure (ie, those who had either viral load ≥400 copies per mL at 12 months or had switched to second-line antiretroviral therapy [ART] as a result of virological failure before 12 months) and controls as those with viral suppression (viral load <400 copies per mL at 12 months) on first-line non-nucleoside reverse transcriptase inhibitor based antiretroviral therapy. We assessed pretreatment drug resistance with Illumina MiSeq next-generation sequencing, using the International Antiviral Society (IAS)-USA mutation list or the Stanford HIV Drug Resistance Database (HIVDB) genotypic sensitivity score. We calculated diagnostic accuracy measures and assessed the odds of virological failure using conditional logistic regression for 1%, 5%, and 10% pretreatment drug resistance detection
thresholds, compared with the conventional 20% or more used in Sanger-based sequencing.

Findings

Paired viral load results before ART and at month 12 of follow-up were available from 1896 participants. We identified 178 patients with virological failure and selected 338 matched controls. We excluded 117 patients from pretreatment drug resistance analysis; therefore, 152 cases of virological failure and 247 controls were included in the final analysis. With the IAS-USA mutation list, at a detection threshold of 20% or more in patients with pretreatment drug resistance, the adjusted odds ratio (OR) for virological failure was 9·2 (95% CI 4·2–20·1) compared with those without pretreatment drug resistance. Lowering the threshold resulted in adjusted ORs of virological failure of 6·8 (95% CI 3·3–13·9) at the 10% threshold, 7·6 (3·4–17·1) at the 5% threshold, and 4·5 (2·0–10·2) at the 1% threshold. Lowering the detection threshold from 20% improved the sensitivity

(ie, ability to identify cases) from 12% (n=18) to 13% (n=19) at detection threshold 10%, to 15% (n=23) at detection threshold 5%, and to 17% (n=26) at detection threshold 1%, but caused a slight reduction in specificity (ie, ability to identify controls) from 98% (n=241) to 96% (n=238) at the 10% threshold, 96% (n=236) at the 5% threshold, and a larger reduction to 92% (n=227) at the 1% threshold. Diagnostic ORs were 5·4 (95% CI 2·1–13·9) at the 20% threshold, 3·8 (1·7–8·6) at the 10% threshold, 3·8 (1·8–8·1) at the 5% threshold, and 2·3 (1·2–4·2) at the 1% threshold. Use of the Stanford HIVDB genotypic sensitivity scores yielded similar ORs for virological failure, sensitivities, specificities, and diagnostic ORs.

Interpretation

Ultrasensitive resistance testing for pretreatment drug resistance improved identification of people at risk of virological failure; however, this came with a reduction in our ability to identify people with viral suppression, especially at very low thresholds. Further modelling is needed to estimate the optimal trade-off for the 5% and 20% thresholds, balancing improved case finding against unnecessary regimen switching.

INTRODUCTION

An estimated 21 million HIV-infected patients were receiving antiretroviral treatment (ART) globally by the end of 2017.[1] Expanded access to life-saving ART is a key achievement in attaining the goal of elimination of HIV as a public health threat by 2030, via the 2020 roadmap of having 90% of all HIV-positive people diagnosed, 90% of those diagnosed on treatment, and 90% of the patients on ART achieving viral suppression.[2] Unfortunately, this progress is being threatened by an emerging epidemic of drug-resistant HIV in low and middle-income countries .[2] Data from WHO show an increase in pretreatment drug resistance, indicating that over one in tenpatients starting ART in several LMIC have drug-resistant HIV associated with non-nucleoside reverse transcriptase inhibitors (NNRTIs).[4] Pretreatment drug resistance is associated with an increased risk of virological failure, impaired immune recovery, accumulation of drug resistance, increased risk of treatment switches and death.[5–8]

In countries with Pretreatment drug resistance prevalence of 10% or more, WHO recommends a shift to alternative, non-NNRTI-based first-line ART (e.g. based on integrase inhibitor or protease inhibitor) or individualized Pretreatment drug resistance testing to guide the choice of first-line treatment.[9] Additionally, continuous resistance surveillance is recommended, as part of the WHO Global Action Plan on HIV drug resistance.[10] However, most low-income and middle-income countries are not able to implement resistance tests largely because of high costs. A technological revolution has led to the development of robust next-generation sequencing technologies with the ability to detect low-frequency minority HIV variants, at increasingly affordable prices.[10] Moreover, several low-cost ultrasensitive point mutation assays are also being evaluated for potential use in low-income and middleincomecountries.[12,13] However, the implementation of these novel tests in low-income and middle-income countries has been slow, partly because of the debate about clinical relevance of drug-resistant minorityvariants.[14] Initial studies were limited by variability in the detection thresholds used to define minority variants of clinical relevance.[15–21] Identification of optimal detection thresholds with clinical relevance is crucial to guide the development and implementation of technologies for resistance testing as part of the public health approach to ART. We did a case-control study nested in a multi-country cohort to assess different detection thresholds for pretreatment drug resistance to optimally predict an increased risk of virological failure on first-line NNRTI based ART.

METHODS

Study design and participants

The PanAfrican Studies to Evaluate Resistance Monitoring Study (PASER-M) was a prospective multicountry cohort established between March, 2007, and November, 2014, which has previously been described in detail.[5] This study was a case-control study nested in the PASER-M cohort, which included patients from 12 clinical sites in Kenya (two sites), Nigeria (one site), South Africa (three sites), Uganda (three sites), and Zambia (three sites). We defined cases as participants with virological failure (ie, who had either viral load ≥400 copies per mL at 12 months or had switched to second-line ART due to virological failure before 12 months). For each case, we randomly selected two controls among participants with viral suppression (viral load <400 copies per mL) on first-line NNRTI-based ART at 12 months, matched for age, country, pre-ART CD4 cell count, and pre-ART viral load. The study was approved by the national and local research ethics committees at the collaborating sites and by the Amsterdam UMC, University of Amsterdam (Amsterdam, Netherlands). Participants provided written informed consent at study enrolment, including for secondary analysis of stored specimens.

Procedures

Stored plasma samples with HIV-1 RNA levels of at least 400 copies per mL, collected at time of ART initiation, were shipped to IrsiCaixa laboratory (Badalona, Spain) for genotyping. Viral amplification was done using a pan-HIV-1 pol in-house assay, as described previously.[22]

Genotyping was done by use of the Miseq (Illumina; San Diego, CA, USA) next-generation sequencing system. DNA libraries were prepared with the Nextera XT DNA Sample Preparation Kit and Nextera XT Index Kit (Illumina), according to manufacturers' instructions. These libraries were then multiplexed into pools of 96 and sequenced with the 500-cycle MiSeq Reagent Kit v.2 (Illumina). Assembly of Miseq sequence reads (FASTQ) and quality assessment were done using PASeq, a freely available automated HIV drug resistance analysis pipeline (IrsiCaixa; Barcelona, Spain).[23] Amino acid variants generated by PASeq were interpreted by use of the 2017 International Antiviral Society-USA (IAS-USA) drug resistance mutation list[24] and the Stanford

HIV Drug Resistance Database (HIVDB; version 8.4).[25] Pretreatment drug resistance was defined in two ways. First, the presence of at least one major IAS-USA

drug resistance mutation associated with any nucleoside reverse transcriptase inhibitor (NRTI) or the NNRTIs nevirapine and efavirenz, and second, genotypic sensitivity scores of 3 or less as per the Stanford HIVDB resistance interpretation: 0 (resistant), 0·5 (low-level and intermediate resistant), and 1 (susceptible and potential low-level resistant). A genotypic sensitivity score of 3 is equivalent to a fully active triple-drug ART regimen.

Statistical analyses

Our sample size estimation assumed that if 15% of controls had minority variants, a minimum sample of 314 patients (105 cases and 209 controls) would provide 90% power to detect an odds ratio (OR) of virological failure of at least 2·5 between cases and controls.

We assessed standard measures of diagnostic accuracy, including sensitivity, specificity, positive and negative predictive values, and diagnostic OR, at different pretreatment drug resistance detection thresholds (1–20%), compared with the Sanger sequencing threshold (≥20%). Diagnostic OR is a measure of the effectiveness of a diagnostic test; in our study, we used this measure to estimate how the different pretreatment drug resistance thresholds discriminated cases from controls, with a low diagnostic OR indicating a low discriminatory ability.

We used conditional logistic regression to assess the association between pretreatment drug resistance and virological failure at month 12. We assessed a priori selected potential confounders (initial NRTI backbone [tenofovir vs abacavir, stavudine, or zidovudine], NNRTI drug [efavirenz or nevirapine], adherence [defined as the 12-month average of 30-day self-reported adherence, fitted as a categorical variable], sex, previous antiretroviral exposure, year of ART initiation, WHO clinical stage, and body-mass index) in univariable models, and if the p value was less than 0·10 we included confounders in the multivariable model. We examined biologically plausible interactions. We adjusted SEs for clustering of observations within sites. We expressed associations as adjusted ORs with 95% CIs. Lastly, we determined the number of patients identified as having pretreatment drug resistance at the different thresholds who would need to be treated with an alternative, fully active first line ART to prevent one case of virological failure (number needed to treat).[26]

We did several additional analyses of the association between pretreatment drug resistance and virological failure. We covered the entire spectrum of pretreatment drug resistance detection thresholds, at decile intervals between 10% and 100% and unit intervals between 1% and 5%. We used a higher viral load

cutoff to define virological failure (≥1000 copies per mL, the WHO definition).[27] We expressed pretreatment drug resistance as mutational load, which we calculated by multiplying the NNRTI mutant frequency in the viral population by the viral load, and expressed as mutant copy number per mL.[18] When multiple drug resistance mutations were observed in one patient, the mutation load was calculated by adding the mutant copy number per mL for each of the detected mutations. We then categorised this value as 0–399 copies per mL, 400–999 copies per mL, or 1000 or more copies per mL.[18] We assessed mean adherence as a potential effect modifier of the association between pretreatment drug resistance and virological failure. All analyses were done with Stata version 12.1.

Role of the funding source

The funders of the study had no role in the study design; data collection, data analysis, data interpretation or writing of the report. The corresponding author had full access to all the data, and had final responsibility for the decision to submit for publication.

RESULTS

Paired viral load results before ART and at month 12 of follow-up were available from 1896 participants. We identified 178 patients with virological failure and selected 338 matched controls (figure 1). Of those with virological failure, 168 (94%) people had a viral load of 400 copies per mL or greater at 12 months and ten (6%) had switched to second-line therapy. We excluded 117 patients from pretreatment drug resistance analysis; therefore, the final analysis included 152 cases of virological failure, of which 57 had one control and 95 had two controls.

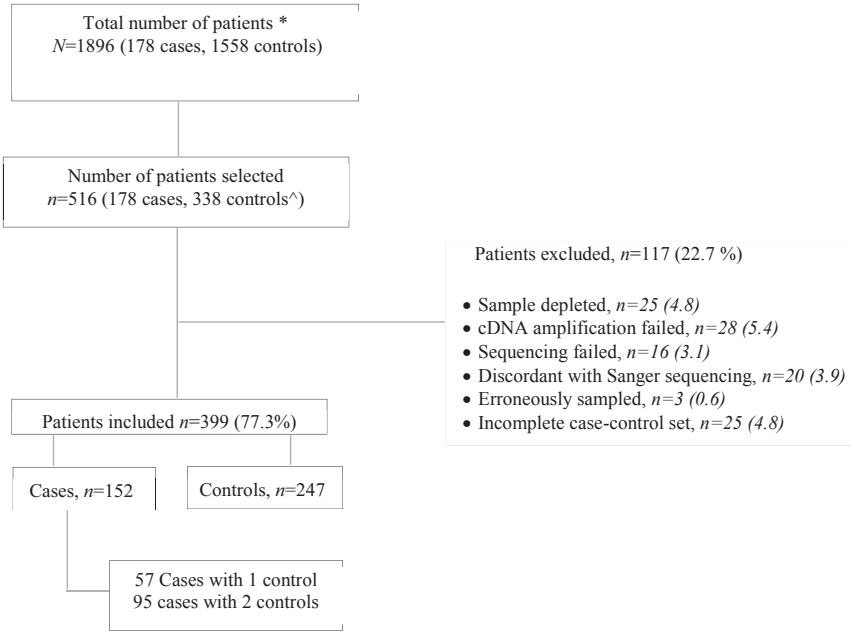

Figure 1: Study profile

* Included only the patients with a viral load test result at 12 months.
^ The 338 included controls were randomly selected from the 1718 controls.

Virological failure cases were more likely to be men, to have a history of previous antiretroviral use, and to have suboptimal adherence (table 1). Other baseline characteristics were evenly distributed between the cases and controls (table 1).

Table 1: Baseline characteristics

Characteristic	All (n=399)	Virological failure Cases n=152	Controls n=247	P-value
Age (years) Median IQR	36.9 (31.0-42.8)	36.7 (30.6-42.6)	37.0 (31.0-42.9)	0.620
Sex, n (%)				**<0.0001**
Female	238 (59.5)	73 (48.0)	165 (66.8)	
Male	161 (40.6)	79 (52.0)	82 (33.2)	
VL (log_{10} cps/mL) Median (IQR)	5.2 (4.5-5.7)	5.2 (4.5-5.7)	5.2 (4.6-5.7)	0.650
CD4 (cells/µL) Median (IQR)	120 (53 -192)	125 (51-199)	117 (58-185)	0.440
WHO clinical staging III/IV n, (%)	252 (62.7)	99 (65.1)	153 (61.9)	0.520
Country, n (%)				0.990
Kenya	89 (22.3)	34 (22.4)	55 (22.3)	
Nigeria	29 (7.3)	12 (7.9)	17 (6.9)	
South Africa	88 (22.1)	32 (21.1)	56 (22.7)	
Uganda	100 (25.1)	39 (25.7)	61 (24.7)	
Zambia	93 (23.3)	35 (23.0)	58 (23.5)	
Type of NNRTI started, n (%)				0.250
EFV	159 (39.9)	86 (56.6)	154 (62.4)	
NVP	240 (60.1)	66 (43.4)	93 (37.7)	
Type of NRTI started, n (%)				0.160
TDF+XTC	141 (35.3)	48 (32.0)	93 (37.7)	
d4T+3TC	97 (24.3)	32 (20.1)	65 (26.3)	
ABC+3TC	6 (1.5)	3 (2.0)	3 (1.2)	
ZDV+3TC	155 (38.9)	69 (45.1)	86 (34.8)	
Previous ARV use, n (%)				**0.040**
No	372 (94.0)*	138 (90.8)	234 (95.9)	
Yes	24 (6.1)	14 (9.2)	10 (4.1)	
Adherence [†], n (%)				**<0.0001**
100%	218 (54.9)**	67 (44.7)	151 (61.1)	
95-99%	103 (25.9)	28 (18.7)	75 (30.4)	
80-95%	36 (9.1)	23 (15.3)	13 (5.3)	
<80%	40 (10.1)	32 (21.3)	8 (3.2)	
HIV-1 subtype, n (%)				0.930
A	114 (28.6)	46 (29.9)	68 (27.5)	
C	191 (47.9)	74 (48.1)	118 (47.8)	
D	42 (10.5)	16 (10.4)	27 (10.9)	
G	11 (2.8)	5 (3.3)	6 (2.4)	
CRF_02AG	9 (2.3)	3 (2.0)	6 (2.4)	
Other recombinants[§]	32 (8.0)	10 (6.5)	22 (8.9)	

Abbreviations: 3TC, lamivudine; ABC, abacavir; d4T, stavudine; TDF, tenofovir, ZDV, zidovudine, XTC, lamivudine/emtricitabine; VL: Viral load;
*Data on previous antiretroviral use were unavailable for three patients.
**Data on adherence were unavailable for two patients.
[†]Mean adherence measured as 30-day self-reported adherence over 12 months.
[§]Other recombinants AD (14), AC (6) AG (4), AG complex recombinants (5) DG (1), CD (2)

Overall, at a detection threshold of 1% or greater, 46 (12%) of 399 patients had pretreatment drug resistance, 36 (9%) associated with NNRTIs and 15 (4%) with NRTIs, based on the IAS-USA list. Similarly, 55 (14%) of 399 patients had a genotypic sensitivity score less than 3, based on the Stanford HIVDB algorithm. The presence of pretreatment drug resistance was more frequent in patients with virological failure than in controls, based on either the IAS-USA list (26 [17%] of 152 vs 20 [8%] of 247; p=0·0006) or Stanford HIVDB algorithm (32 [21%] of 152 vs 23 [9%] of 247; p=0·001; figure 2)

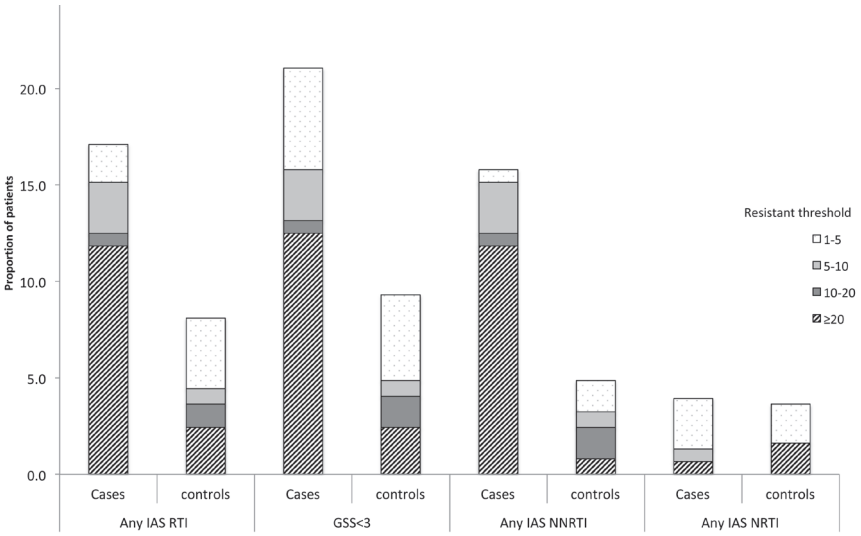

Figure 2: Comparative analysis of pre-treatment drug resistance between cases and controls at different detection thresholds

Drug resistance was defined as i) Presence of any IAS-USA mutation for NRTIs and efavirenz/nevirapine NNRTIs ii) Genotypic sensitivity score <3 according to Stanford HIVdb.

On the basis of the IAS-USA list, lowering of the detection threshold from 20% improved the sensitivity (ie, ability to identify cases) from 12% (n=18) to 13% (n=19) at detection threshold 10%, to 15% (n=23) at detection threshold 5%, and to 17% (n=26) at detection threshold 1%. This improvement in sensitivity came with a slight reduction in specificity (ie, ability to identify controls) from 98% (n=241) to 96% (n=238) at the 10% threshold, to 96% (n=236) at the 5% threshold, and a larger reduction to 92% (n=227) at the 1% threshold (table 2). Across all realistic scenarios for pretreatment drug resistance prevalence in a given patient

population (0–20%), positive predictive value was highest for the 20% detection threshold and lowest for the 1% detection threshold, but these differences were not statistically significant (figure 3; table S1). Negative predictive value did not vary between the detection thresholds (figure 3). Diagnostic OR was highest at the 20% detection threshold, and decreased as the threshold was lowered (table 2). The number needed to treat to prevent one case of virological failure was lowest at the 20% detection Similar findings were observed with the Stanford HIVDB algorithm (table 2).

Table 2: Diagnostic accuracy measures of different detection thresholds of pre-treatment drug resistance

Cut-off	Sensitivity % (95%CI)	Specificity % (95%CI)	DOR (95%CI)	*NNT
IAS $_{NRTI/EFV/NVP}$				
≥20%	12 (7-18)	98 (95-99)	5.4 (2.1-13.9)	3
≥10%	13 ((8-19)	96 (93-98)	3.8 (1.7-8.6)	4
≥5%	15 (10-22)	96 (92-98)	3.8 (1.8-8.1)	4
≥1	17 (12-24)	92 (88-95)	2.3 (1.2-4.2)	6
GSS HIVDB <3.0				
≥20%	13 (8-19)	98 (95-99)	5.7 (2.2-14.7)	5
≥10%	13 (8-19)	96 (93-98)	3.6 (1.6-7.9)	6
≥5%	16 (10-23)	95 (92-98)	3.7 (1.8-7.6)	6
≥1%	21 (15-29)	91 (86-94)	2.6 (1.5-4.6)	9

Abbreviations: IAS, International Antiviral Society mutation list; DOR, diagnostic odds ratio; GSS, genotypic sensitivity scores

Sensitivity denotes the proportion of cases (patients with virological failure) who were identified as having PDR; Specificity denotes the proportion of controls (patients without virological failure) who were identified as not having PDR; DOR diagnostic odds ratio is a measure of the effectiveness of the diagnostic tests; It describes the odds of having PDR among cases relative to having PDR in the controls, expressed as [(sensitivity * specificity) / (1-sensitivity) * (1-specificity)].

*NNT: Number of patients detected with PDR at the different PDR mutant detection thresholds, who needs to be treated with a fully active ART to prevent one case of virological failure. NNT was calculated as $\left(\frac{1}{OR-1} * \pi o + \frac{OR}{(OR-1)*(1-\pi 0)}\right)$ where OR is the odds ratio and is the rate of exposure in the controls (rates of virological failure among persons without PDR at the selected cut-off estimated from the full cohort) *rounded off to the next whole number

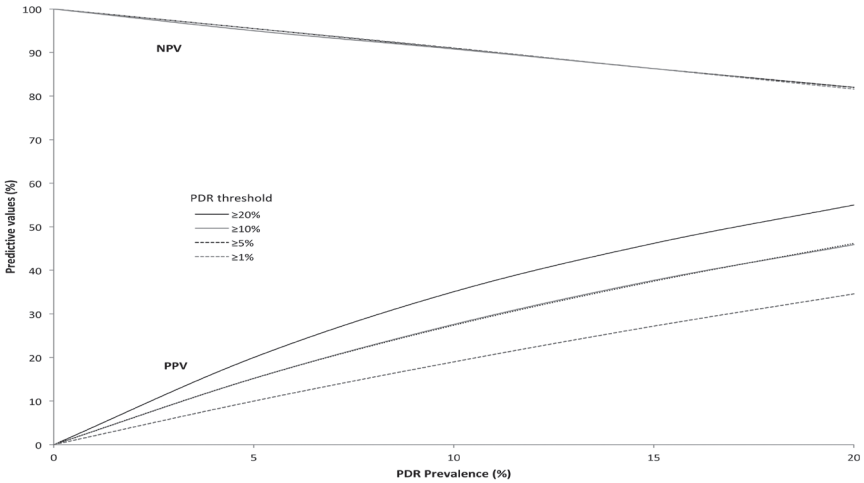

Figure 3: Predictive values to identify patients at risk of virological failure for different PDR thresholds across different PDR prevalences

Figure shows the PPV and NPV as a function of different PDR prevalences.
Positive predictive value (PPV); denotes the proportion of patients identified as having PDR who are predicted to have virological failure, Negative predictive value (NPV); denotes the proportion of patients identified as not having PDR who are predicted to have virological suppression

With the IAS-USA mutation list, most pretreatment drug resistance mutations occurred at the 20% or more threshold (figure 2). At this threshold, in patients with pretreatment drug resistance, the adjusted OR for virological failure was 9·2 (95% CI 4·2–20·1) compared with those without pretreatment drug resistance. Lowering the threshold resulted in adjusted ORs of

virological failure of 6·8 (95% CI 3·3–13·9) at the 10% threshold, 7·6 (3·4–17·1) at the 5% threshold, and 4·5 (2·0–10·2) at the 1% threshold (table 3). Using the Stanford HIVDB algorithm, we observed a similar effect of pretreatment drug resistance on virological failure at the different thresholds, but with slightly lower strength of association (table 3).

Table 3: Effect of PDR on 12 months virological failure: Determination of clinically relevant resistance thresholds

Characteristic	Participants with PDR (n)	Cases with PDR (n)	Unadjusted OR (95%CI)	P-value	Adjusted OR± (95%CI)	P-value
IAS $_{NRTI/EFV/NVP}$						
≥20%	24	18	6.7 (2.3-19.1)	0.001	9.2 (4.2-20.1)	<0.0001
≥10%	28	19	4.1 (1.6-10.0)	0.002	6.8 (3.3-13.9)	<0.0001
≥5%	34	23	4.7 (2.1-10.6)	<0.0001	7.6 (3.4-17.1)	<0.0001
≥1%	46	26	2.3 (1.3-4.4)	0.007	4.5 (2.0-10.2)	<0.0001
GSS <3.0						
≥20%	25	19	5.7 (2.4-13.7)	< 0.0001	5.4 (2.1-14.1)	0.001
≥10%	30	20	3.3 (1.3-8.4)	0.013	4.3 (1.9-9.6)	<0.0001
≥5%	36	24	3.8 (1.7-8.3)	0.001	4.6 (2.2-9.7)	<0.0001
≥1%	55	32	2.8 (1.4-5.5)	0.004	3.3 (1.5-7.5)	0.004

IAS, International Antiviral Society mutation list; GSS, genotypic sensitivity scores
±Adjusted for sex, type of NNRTI and NRTI initiated, adherence and prior ARV exposure

23 (96%) of 24 NNRTI drug resistance mutations among patients with virological failure occurred at frequencies of between 5% and 100%, and in controls ten (83%) of 12 NNRTI drug resistance mutations occurred at frequencies of between 1% and 10%. NRTI mutations mainly occurred at the 5% or less threshold inpatients with virological failure (five [83%] of six) and controls (five [56%] of nine; figure 4).

Overall, Lys103Asn/Ser, Gly190Ala/Ser, and Tyr181Cys were the most frequently observed mutations, occurring mainly among patients with virological failure at frequencies of between 5% and 100% (figure 4). By contrast, only six controls had Lys103Asn mutation, five (83%) of which occurred at the detection threshold of 10% or less. One control had Gly190Ala mutation (occurring at the 90% threshold) and one control had Tyr181Cys mutation (occurring at the 10% threshold). Val106Ala/Met, Tyr188Cys/Leu, and Lys65Arg mutations occurred exclusively among patients with virological failure, whereas Asp67Asn, Lys70Arg, Leu74Val, and Lys219Gln/Glu mutations occurred only among controls.

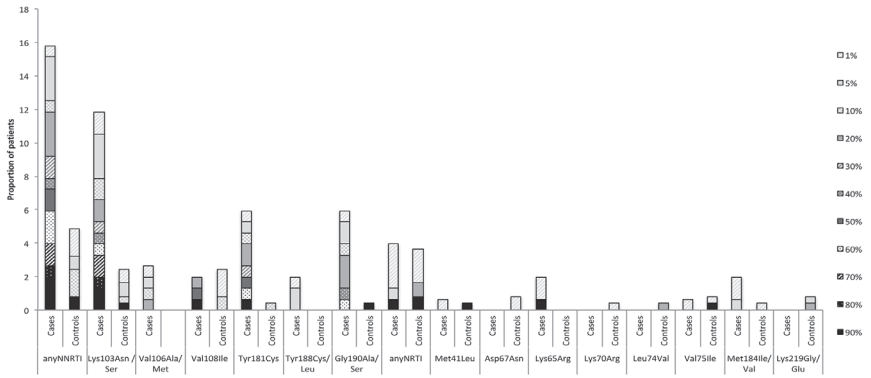

Figure 4: Frequency and pattern of pretreatment HIV drug resistance mutations at different detection thresholds

We did several additional analyses. We assessed the effect of pretreatment drug resistance (IAS-USA list) on virological failure across a wider spectrum of detection thresholds. Overall, a 30% threshold had the highest adjusted OR (14·2, 95% CI 3·5–57·9; table S3), which decreased at lower thresholds. When using a higher cutoff to define virological failure (1000 copies per mL), the strength of the association between pretreatment drug resistance and virological failure was similar at the 20% and 5% thresholds (table S4). When we expressed pretreatment drug resistance as mutational load, the effect on virological failure was significant only at a higher copy number (≥1000 copies per mL; table S5). We found evidence that the association between pretreatment drug resistance and virological failure was significantly modified by the level of adherence, with a greater effect size in highly adherent patients than in poorly adherent patients (table S6).

DISCUSSION

Ultrasensitive resistance testing for pretreatment drug resistance increased our ability to identify people at risk of virological failure (sensitivity) compared with the conventional 20% detection threshold used in Sanger sequencing. However, this increased sensitivity came with a reduction in ability to identify people with viral suppression (specificity), especially at very low (1%) detection thresholds. In an extended analysis, which also accounted for pretreatment drug resistance prevalence, we found that the diagnostic performance at 5%, 10%, and 20%

detection thresholds did not show any statistically significant differences, in terms of positive and negative predictive values, diagnostic OR, and number needed to treat. Our data also suggest that further reduction to a very low (1%) threshold could lead to high misclassification of patients with viral control as being at risk of virological failure (false positives).

The real-world implications of improved detection of patients with virological failure by use of a more sensitive detection threshold needs to be balanced against the probable increase in false positives. In a hypothetical example of a population of 100 000 patients starting ART, in which we assume that pretreatment drug resistance prevalence is 10%, use of a 1%, 5%, or 10% pretreatment drug resistance detection threshold rather than a 20% threshold could result in detection of an additional 500 (42% increase), 250 (25% increase), or 100 (8% increase) patients respectively who are likely to have virological failure. However, this comes at the expense of an additional 5400 (300% increase), 1800 (100% increase), or 1800 (100% increase) patients respectively who are likely to be misclassified as being at risk of virological failure (table S2). Consequently, the use of a very low (1%) detection threshold could result in higher number of patients unnecessarily being started on more costly, alternative, first-line ART regimens. Comprehensive mathematical modelling will be needed to project the optimal trade-off for the 5% and 20% threshold, and the clinical and cost implications at the population level.

Although there is no consensus, some studies have shown the clinical impact of minority variants in both treatment naive and experienced populations.[17,18,21] A key limitation in previous studies was the exclusion of patients with major circulating variants (drug resistance mutations circulating at ≥20% of the virus population), and therefore an inability to determine an optimum detection threshold for use with ultrasensitive resistance assays. Our study analysed the entire spectrum of thresholds for the detection of drug-resistant virus populations. Although most clinically relevant mutations exist as major variants, we showed that use of more sensitive thresholds might improve detection of patients who are likely to have virological failure. These findings corroborate a previous report from a nationwide cohort study in Mexico,[6] which suggested that lowering the detection threshold (ie, including minority variants) to 5% could improve the ability to predict patients with virological failure, but that study did not express any other diagnostic accuracy measures.

Although some studies have proposed use of very low thresholds to detect minority variants, ou r findings suggest that this could result in an increased proportion of patients being misclassified as at risk of virological failure, who would in fact maintain good viral control.[18,21] In our study, the use of a 1% threshold led to a false positive rate of 8%, compared with 4% at the 5% threshold and 2% at the 20% threshold. From our data, this rate was estimated as six to nine patients for every case of virological failure averted at the 1% threshold compared with three to six patients at the 5% and 20% thresholds. Previous studies suggested that the clinical relevance of pretreatment drug resistance could be dependent on the specific mutations detected and the frequency at which they occur.14,20,21 As expected, we observed that NNRTI mutations were the main drivers of virological failure and occurred mainly at thresholds of 5% or greater, but at lower prevalence and lower thresholds in controls. The NNRTI signature mutations (Lys103Asn, Val106Ala/Met, Tyr188Cys/Leu, and Gly190Ala), which cause intermediate-level to high-level resistance, occurred mainly in patients with virological failure. However, NRTI resistance mutations generally occurred infrequently, but at comparably lower thresholds in both patients with virological failure and controls. The NRTI mutations Asp67Asn, Lys70Arg, Leu74Val, and Lys219Gln/Glu occurred only among controls. These findings suggest that the risk of virological failure due to pretreatment drug resistance is driven mainly by preexisting NNRTI mutations and indicate a minimal role of NRTI resistance. Moreover, these findings could be used to inform the type of drug resistance mutations to be included in point mutation assays, but further studies are needed to determine mutation-specific thresholds.

Adoption of HIV drug resistance tests in low-income and middle-income countries has been hampered by the high costs associated with Sanger-based sequencing methods. However, the sequencing landscape has changed over the past decade, with the rise of more affordable next-generation sequencing technologies, which can be used to facilitate wider access to HIV drug resistance testing in resource-limited settings.[13] Apart from the lack of consensus on a clinically relevant threshold to detect resistance, implementation of next generation sequencing assays had been restricted by the need for complex bioinformatics analysis. However, open source and automated easy to use analysis tools are becoming widely available, including the one used in this study (PASeq).[23] Use of next-generation sequencing might in the short term be cost-effective only for high throughput laboratories, but there are several potential low-cost ultrasensitive

point mutation assays being evaluated for use at low throughput facilities.[12] With emerging HIV drug resistance in low-income and middle-income countries,[3] there will be an increased need for HIV drug resistance surveys in sub-Saharan Africa, as advocated by WHO,[4,9] and for individualised testing in patients with ART failure to guide drug choices.[9] Low cost assays could help facilitate these activities and the subsequent optimisation of first-line regimens.

The main strength of our study was that cases and controls were derived from a well characterised, multicountry prospective cohort in a highly relevant setting, in which the impact of increasing NNRTI-associated pretreatment drug resistance is of growing concern. Additionally, the nested case-control study design allowed for control of the most important confounders, and we could correlate results from next-generation sequencing with Sanger sequencing. Our main findings were proven to be robust in additional analyses using different definitions for virological failure and pretreatment drug resistance. To our knowledge, this is the first study to assess the clinical significance of drug resistance across the entire spectrum of resistance detection thresholds and also to contribute important evidence to support the conventional threshold for Sanger-based sequencing assays.

Our study has some limitations. First, our findings mainly applied to patients starting on NNRTI-based first-line therapy, which has been the core drug class of WHO-recommended regimens for first-line therapy and prevention of mother-to-child transmission of HIV in low-income and middle-income countries for the past two decades. Although some low-income countries will transition towards rollout of dolutegravir, an integrase inhibitor, in first-line ART in the coming years because of price reductions,[28] the NNRTI drug class is expected to remain in use as standard first-line therapy in many other low-income and middle-income countries, especially in women of child-bearing age (because of safety concerns for use of dolutegravir in this group),[29,30] confirming the relevance of our study findings for current and future practice. Some evidence shows selection of minority variants, which might affect the predicted susceptibility of dolutegravir, especially in integrase strand transfer inhibitor-experienced patients.[31] More studies are needed to understand the clinical relevance of integrase strand transfer inhibitor minority variants. Second, residual unmeasured or unknown confounding factors could have influenced some of the findings. Third, because of the observational design of the study, the associations found

do not necessarily indicate causality, and case-control studies do not allow for calculation of absolute risk. Fourth, although the patients came from routine ART programmes in different geographical settings, the study population is not necessarily representative of all people with HIV/AIDS in the region studied and caution is warranted when extrapolating results to different subpopulations or countries.

In conclusion, our findings show that incorporating minority variants might improve the prognostic value of HIV drug resistance tests, whereas very low thresholds compromise test specificity. Further modelling studies are needed to estimate the optimal trade-offs and project overall implications for the different thresholds.

Competing interests

All authors declare that they have no conflict of interest.

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Authors' contributions

TFRW is the PASER principal investigator. RLH, CK and TFRW established the cohort and supervised data collection. RLH, TFRW and RP conceived the study. SCI, MN-J, MC and MP perfomed the laboratory testing and data analysis. SCI, RLH, TFRW and RP drafted the manuscript. All authors provided valuable input to interpretation of the data and critically reviewed the paper for important intellectual content. All authors reviewed and approved the final version of the manuscript.

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Table S1: Predictive values of virological failure for different PDR detection thresholds

PDR prevalence	Positive predictive value (95%CI)				Negative predictive value (95%CI)			
	PDR detection threshold				PDR detection threshold			
	1%	5%	10%	20%	1%	5%	10%	20%
5%	10 (6-16)	15 (8-26)	15 (8-28)	20 (9-39)	96 (95-96)	96 (95-96)	95 (95-96)	96 (95-96)
10%	19 (12-29)	27 (16-43)	28 (15-45)	35 (18-57)	91 (90-92)	91 (90-92)	91 (90-91)	91 (90-91)
15%	27 (18-39)	38 (23-54)	38 (22-57)	46 (26-68)	86 (85-87)	86 (86-87)	86 (85-87)	86 (86-87)
20%	35 (23-48)	46 (30-63)	46 (29-65)	55 (33-75)	82 (80-83)	82 (81-83)	82 (81-83)	82 (81-83)

Abbreviations: PDR, pretreatment resistance;
PDR was determined by the International Antiviral Society-USA mutation list;
Positive predictive value (PPV) denotes the proportion (%) of patients identified as having PDR who are predicted to have virological failure
Negative predictive value (NPV) denotes the proportion (%) of patients identified as not having PDR who are predicted to have virological suppression.

Table S2: Simulation of detected cases of virological failure versus false-positives for different PDR detection thresholds

PDR prevalence	Case finding (n, %)				False positive, n (%)			
	PDR detection threshold				PDR detection threshold			
	1%	5%	10%	20%	1%	5%	10%	20%
5%	250 (42%)	150 (25%)	50 (8.3%)	600	5700 (300%)	1900 (100%)	1900 (100%)	1900
10%	500 (42%)	300 (25%)	100 (8.3%)	1200	5400 (300%)	1800 (100%)	1800 (100%)	1800
15%	750 (42%)	450 (25%)	150 (8.3%)	1800	5100 (300%))	1700 (100%)	1700 (100%)	1700
20%	1000 (42%)	600 (25%)	200 (8.3%)	2400	4800 (300%))	1600 (100%)	1600 (100%)	1600

These estimates were based on a hypothetical population of 100,000 patients starting ART. Numbers (delta %) of detected cases with virological failure and false-positives were calculated for each PDR detection threshold, relative to the 20% threshold. False-positive refers to misclassifying patients with viral control as being at risk of VF.

Abbreviations: PDR, pretreatment resistance;

Table S3: Clinically relevant detection thresholds across a wider spectrum of detection thresholds (additional analysis #1)

Characteristic	Participants with PDR	Cases with PDR	Unadjusted OR (95%CI)	P-value	Adjusted OR± (95%CI)	P-value
IAS $_{NRTI/EFV/NVP}$						
≥90%	7	3	1.0 (0.2-5.0)	1.000	4.9 (0.4-64.0)	0.220
≥80%	8	4	1.6 (0.3-7.4)	0.550	4.9 (0.4-63.7)	0.220
≥70%	10	6	2.8 (0.9-9.4)	0.090	7.7 (1.1-53.1)	0.040
≥60%	13	9	6.1 (1.8-20.7)	0.003	8.9 (1.5-50.9)	0.010
≥50%	15	11	7.7 (2.2-26.4)	0.001	13.3 (3.0-60.0)	0.001
≥40%	16	12	8.7 (3.0-25.4)	<0.0001	14.1 (3.5-56.6)	<0.0001
≥30%	18	14	9.7 (3.6-26.6)	<0.0001	14.2 (3.5-57.9)	<0.0001
≥20%	24	18	6.7 (2.3-19.1)	<0.0001	9.2 (4.2-20.1)	<0.0001
≥10%	28	19	4.1 (1.6-10.0)	0.002	6.8 (3.3-13.9)	<0.0001
≥5%	34	23	4.7 (2.1-10.6)	<0.0001	7.6(3.4-17.1)	<0.0001
≥4%	34	23	4.7 (2.1-10.6)	<0.0001	7.6(3.4-17.1)	<0.0001
≥3%	36	23	3.5 (1.6-7.6)	0.001	6.8 (3.6-13.0)	<0.0001
≥2%	37	23	3.2 (1.7-6.0)	<0.0001	5.6 (3.0-10.6)	<0.0001
≥1%	46	26	2.3 (1.3-4.4)	0.007	4.5 (2.0-10.2)	<0.0001

Abbreviations: IAS, International Antiviral Society mutation list; OR, odds ratio; VF, virological failure
The analysis included 399 patients; 152 cases with 247 controls
±Adjusted for sex, type of initial NNRTI and NRTI, adherence and prior ARV exposure

Table S4: Clinically relevant detection thresholds, using a higher viral load cut-off to define virological failure (≥1000 cps/ml) (additional analysis #2)

Characteristic	Participants with PDR	Cases with PDR	Unadjusted OR (95%CI)	P-value	Adjusted OR ± (95%CI)	P-value
IAS						
≥20%	22	16	5.9 (2.1-16.9)	0.001	8.7 (3.9-19.6)	<0.0001
≥10%	25	17	4.2 (1.6-11.2)	0.004	7.1 (3.7-13.8)	<0.0001
≥5%	30	20	4.6 (1.9-11.0)	0.001	8.3 (3.6-18.8)	<0.0001
≥1%	40	23	2.6 (1.3-5.3)	0.010	5.5 (2.7-11.4)	<0.0001
GSS HIVDB <3.0						
≥20%	23	18	6.8 (2.1-21.4)	0.001	5.7 (1.6-20.8)	0.008
≥10%	28	19	3.6 (1.3-10.2)	0.020	4.6 (1.7-12.5)	0.003
≥5%	33	22	3.9 (1.7-9.2)	0.002	5.0 (1.9-13.1)	0.001
≥1%	53	30	2.8 (1.3-5.7)	0.006	3.4 (1.3-8.9)	0.010

Abbreviations: IAS, International Antiviral Society mutation list; GSS, genotypic sensitivity scores; OR, odds ratio; VF, virological failure
The analysis included 338 patients; 129 cases with 209 controls
±Adjusted for sex, type of initial NNRTI and NRTI, adherence and prior ARV exposure

Table S5: Clinically relevant detection thresholds, using mutational load to define PDR (additional analysis #3)

Characteristic	Participants with PDR	Cases with PDR	Unadjusted OR (95%CI)	P-value	Adjusted OR ± (95%CI)	P-value
Mutational load						
<400 cps/ml	365	129			1.0	
400-999 cps/ml	1	0	N/A	N/A	N/A	N/A
≥1000 cps/ml	33	23	2.3 (1.5-3.7)	<0.0001	2.9 (1.5-5.4)	0.001

Abbreviations: PDR, pre-treatment drug resistance; VF, virological failure;
The analysis included 399 patients; 152 cases with 247 controls
PDR and mutational load were assessed based on IAS-USA mutation list
±Adjusted for sex, type of initial NNRTI and NRTI initiated, WHO clinical stage, BMI, calendar year of ART initiation, adherence and prior ARV exposure

Table S6: Effect modification of the association between PDR and virological failure (additional analysis #4)

Characteristic	With PDR	PDR in cases	§Adjusted OR (95%CI)	P-value
Effect modification				
PDR≥5% + adherence				0.009±
PDR≥20% + adherence				0.006±
Stratified analysis				
PDR ≥5% + ≥95% adherence	31	21	8.2 (2.4-27.2)	0.001
PDR ≥5% + <95% adherence	3	1	1.0*	
PDR ≥20% + ≥95% adherence	21	16	14.3 (3.6-56.5)	<0.0001
PDR ≥20% + <95% adherence	3	1	1.0*	

Table shows the association of PDR and virological failure stratified by the level of adherence.
We assessed potential effect modifiers of the association between PDR with VF.
±P for interaction; * Low numbers limited the analysis.
§Adjusted for sex, type of initial NNRTI and NRTI and prior ARV exposure
The analysis included 399 patients; 152 cases with 247 controls.

Chapter 17

Summary and general discussion

SUMMARY AND GENERAL DISCUSSION

The last 15 years have witnessed an unprecedented scale-up of antiretroviral treatment (ART) in sub-Saharan Africa. Approximately 60% of the 25.7 million HIV-infected persons living in Africa were estimated to be on treatment by the end of 2017.[1] The improved access to life-saving treatment has been accompanied by significant reductions in HIV-related morbidity, mortality as well as incidence. [1] By 2017, annual HIV-related deaths had dropped by nearly 30% from an estimated 1.3 million a decade ago.[1,2]

With increased access to ART, the global attention has been shifting from the initial goal of slowing progression to AIDS and death, to that of achieving successful long-term clinical outcomes, improving quality of life and preventing new infections. To achieve these goals, UNAIDS in 2014 set global targets for having 90% of people living with HIV knowing their status, 90% of those infected receiving ART, and 90% of those on ART having sustained viral suppression.[3] Achieving these targets by 2020 and increasing to 95%, 95%, 95% thereafter is projected to result in control of the HIV epidemic by 2030.[3]

There are multiple factors challenging the attainment of these targets. The current thesis concentrates on the challenge for achieving the third '90' goal for viral suppression with the specific focus on adherence, drug resistance and lack of better molecular diagnostics in sub-Saharan Africa. The studies in this thesis were conducted as part of the Pan-African African Studies to Evaluate Resistance in Africa (PASER-M), a multi-center cohort in six African countries; Kenya, Nigeria, South-Africa, Uganda, Zambia and Zimbabwe.[4]

The aims of the studies described in the thesis were to identify determinants of long-term sustained viral suppression, to describe the prevalence of pretreatment drug resistance (PDR) among HIV-infected infants and to assess novel, affordable diagnostics for improving ART monitoring in sub-Saharan Africa.

PART I: DETERMINANTS OF LONG-TERM SUSTAINED VIRAL SUPPRESSION

In Part I, we assessed factors that hinder or facilitate the attainment of sustained long-term viral suppression. In **Chapter 2,** we assessed the risk of prior ARV use on virological response and the extent to which this effect was mediated through PDR. Findings from the WHO 2017 HIV drug resistance (HIVDR) report, estimate that 1 in 5 patients initiating first-line treatment in sub-Saharan Africa have a history of prior ARV use; this includes re-starters who have disengaged from care, or mothers who have used short-course antiretrovirals (ARVs) for prevention of mother to child transmission (PMTCT) or those failing on pre- and post-exposure prophylaxis.[5] Such patients may be at risk of having PDR, which may impair response to the standard first-line treatment. In this study, 4.5% of the 2737 participants initiating first-line ART in the PASER-M cohort, had a history of prior ARV use which predisposed them to a 7.2 fold risk of carrying PDR and 3.0-fold risk for developing virological failure when compared to antiretroviral naïve participants. Intervening on PDR would eliminate 50% of the effect of prior ARV use on virological failure. These findings highlighted the need for using a differentiated non-nucleoside reverse transcriptase inhibitor (NNRTI) based first-line ART for patients with prior ARV use. Based on the evidence from this and other studies, WHO issued an interim guideline in 2017 recommending the use of a non-NNRTI-based first-line for patients with a history of prior ARV use.[6]

In **Chapter 3** we assessed the impact of PDR on different NNRTI-based first-line ART within the PASER-M cohort. Of the 2,737 participants initiating first-line ART, 1,941 had data on PDR and 12-month viral load. Initial regimens contained tenofovir+lamivudine/emtricitabine (xtc) (33%), with efavirenz (27.3%) or nevirapine (5.7%), or a non-tenofovir, thymidine analogue backbone+xtc (67%), with efavirenz (29.8%) or nevirapine (37.1%). 1838 (94.7%) patients had no PDR, 79 (4.1%) had NNRTI-PDR only, 44 (2.3%) had NRTI-PDR and 24 (1.2%) had dual-class NNRTI+NRTI-PDR. Virological failure was present in 335 (17.3%), 199 (10.3%) and 172 (8.9%) participants at viral load of ≥50, 400 and 1000 cps/ml thresholds, respectively. Participants who had PDR and received non-tenofovir/xtc with efavirenz or nevirapine, had an increased risk of virological failure, compared to those without PDR. However, this risk was not increased for participants who received tenofovir/xtc/efavirenz, whereas there was a borderline association for participants who received tenofovir/xtc/nevirapine. Participants with NNRTI-

PDR who received a tenofovir-containing regimen had an increased risk of virological failure at the VL≥1000 cps/ml threshold (with borderline statistical significance p=0.073), and the risk was not increased at the ≥50 and ≥400 cps/ml thresholds. These findings suggest that NNRTI-PDR may impact less on tenofovir/xtc/efavirenz than on thymidine analogue-based regimens especially, with nevirapine. More data is needed to understand the extent of genetic barrier of tenofovir/xtc/efavirenz on patients initiating treatment with PDR.

In **Chapter 4** we assessed the durability of first-line treatment in a cohort of 955 patients in western Kenya.[7] Within a median follow-up period of 10.7 months, 18.7% of the patients had modified their treatment regimen, mainly due to drug toxicities. Risk factors for ART modifications were: initiating ART with advanced disease stage (WHO stage III/IV), or low CD4 counts, being older, use of stavudine (d4T) and high baseline weight. Use of tenofovir (TDF) and zidovudine (ZDV) were associated with 49% and 40% reductions in the risk of treatment modifications. These results were not surprising, as d4T has long been known to be intolerable and treatment guidelines from 2010 had already recommended its phase-out.[8–10] However, due to costs, logistics and limited available alternatives, there was a slow phase-out of d4T-based regimens in some countries.[11] The experience with d4T highlights the need for increased pharmacovigilance, wide-access and rapid-transition to better, more tolerable drugs to increase the durability of the limited drug options available in Africa.

The ability to achieve sustained viral suppression is dependent on sustaining a lifelong optimal adherence. In **Chapter 5**, we described the findings of a qualitative study using in-depth interviews with care providers to assess the determinants of long-term antiretroviral treatment adherence in adolescents and adults in Uganda.[12] Adolescents are a particularly challenging group with worse treatment outcomes than either adults or children.[13–16] Determinants of poor adherence among adolescents included unstructured treatment holidays, delayed disclosure of HIV status by caretakers, stigma, which was mainly experienced in boarding schools, diminishing or lack of clinical support for young adults during transition to adult-based care and declining peer-to-peer support group activities. The main barrier for adherence in adults was difficulties in acceesing treatment among temporary economic migrants. Common barriers for adherence in adults and adolescents were challenges with disclosure in intimate relationships; treatment-related factors including side effects, health

system related factors including, supply of single tablets in place of fixed-dose combined drugs, supply of drug brands with unfavourable taste and missed opportunities for counselling due to shortage of staff. These findings indicate the need for adherence interventions tailored to emerging life-course challenges in long-term treated patients. Moreover, programs should endeavor to support adolescents living with HIV in particular by offering support to caretakers for early and gradual disclosure of HIV status, supportive gradual transition to adult-based care as well as maintaining functional peer-support groups.

In **Chapter 6,** we describe an emerging concern for exhaustion of therapy options for patients failing second-line ART in Kenya.[17] In this nationwide cross-sectional study, 1 in 4 patients failing second-line ART had exhausted all the available treatment options indicating the need for increased access to third-line drugs in these settings. Alternative third-line drugs are prohibitively expensive, typically costing between 6 and 14 times more than first- and second-line therapies, and are therefore out of reach for ART programs in low- and middle-income countries (LMICs).[15]

In **Chapter 7** we assessed primary resistance to integrase strand transfer inhibitors (INSTIs) across the major HIV-1 subtypes circulating in sub-Saharan Africa.[18] Of 425 genotypes isolated from viral isolates among participants in the PASER-M cohort, 48.7% were subtype C, 28.5% A, 10.1% D, 2.8% G, and 9.9% were recombinants. Major INSTI resistance mutations were detected only at <20% threshold, at a prevalence of 2.4% (2.5% in subtype A, 2.4% C, 0% D, 8.3% G and 2.4% in recombinants) and included T66A/I (0.7%), E92G (0.5%), Y143C/S (0.7%), S147G (0.2%) and Q148R (0.5%). Accessory mutations occurred at a prevalence of 15.1% at the ≥20% threshold (23.1% in subtype A, 8.7% C, 11.6% D, 25% G and 23.8% in recombinants), and included L74I/M (10.4%), Q95K (0.5%), T97A (4%), E157Q (0.7%) and G163R/K (0.7%). Overall major resistance mutations were rare and only occurred at low-level frequencies, which suggests that these agents are likely to be effective across the diverse subtypes in this region.

Chapter 8 is a Viewpoint in which we presented some cautious notes against presenting dolutegravir (DTG) as an overall solution to the rise in HIVDR in LMICs. First, current safety concerns for DTG use in women of reproductive age may imply the need for an alternative solution to HIVDR in this group.[19] Second, pre-existing resistance to the co-administered NRTI backbone may reduce effectiveness and durability of DTG, potentially further augmented when access to viral load tests is

limited. Third, there is limited information on the genetic correlates of resistance to DTG, particularly in patients infected with HIV-1 non-B subtypes. Finally, clinical management of patients who experience virological failure on a DTG-based regimen will pose challenges due to the uncertainty of whether DTG resistance has actually developed and switching is needed or whether improved adherence is sufficient. Overall, we emphasize the need to address these concerns so as to consolidate the expected gains from widespread introduction of DTG in LMICs.

PART II: BURDEN OF PDR IN HIV-INFECTED INFANTS

The HIV response in children has generally lagged behind the progress in adults. In particular, HIV-infected children have comparatively fewer ART options, low treatment coverage and a high risk of drug resistance arising from exposure to the prophylactic ARVs used for PMTCT. In Part II we assessed the burden of PDR in infants and potential mitigation strategies.

In **Chapter 9** we described the emergence of K65R, a multi-NRTI resistance mutation, in breastfeeding children exposed to maternal PMTCT regimens (ZDV plus lamivudine (3TC) or nevirapine (NVP) /nelfinavir not previously known to select for this mutation.[20] We included 24 infants who acquired HIV-infection during the breastfeeding period (up to 6 months post-partum) in the Kisumu breast-feeding PMTCT clinical trial in Kenya. Overall, 6 of the 24 (25%) infants developed the K65R mutation within six months. None of the mothers at delivery or the infants with a genotype at first test of positivity had the K65R mutation, suggesting that it was likely not transmitted but acquired from exposure to a sub-optimal maternal 3TC regimen. This study highlighted the need for elucidation of the mechanism of K65R selection in presence of sub-optimal levels of 3TC.

In **Chapter 10** we described a high prevalence of PDR in infants <18 months in a nationwide survey in Nigeria.[21] Overall, 48% of 430 infants had drug resistance mutations (DRMs), predominantly to NNRTIs (45%) and 20% had multi-class resistance to both NNRTIs and NRTIs. The prevalence of PDR among infants with exposure to PMTCT drugs (204) was 57% and in the unexposed infants was 34% (132). These findings indicate an urgent need to adopt the WHO guidelines recommending a protease-inhibitor (PI)-based first-line for all infants regardless of PMTCT history in LMICs.

In **Chapter 11**, we discussed potential strategies to prevent the emergence of PDR in infants.[22] Although WHO recommends the use of PI-based first-line in children[23], uptake is hindered by costs and logistics constraints and thus up to 76% are still placed on NNRTI-based regimens, despite the high risk of having NNRTI-PDR.[24] Preventing the emergence of PDR is vital, particularly with the underlying limited pediatric treatment options globally available. Transmission of HIVDR from mother-to-child could be prevented by increasing the frequency of monitoring maternal viral-loads during prepartum and throughout breastfeeding, complemented with enhanced adherence interventions and use of INSTI-based regimens for those experiencing treatment failures so as to facilitate rapid viral suppression. In addition, the use of triple-ART prophylaxis, or prophylactic regimens with a high genetic barrier in infants could further help prevent PDR acquired from exposures to sub-therapeutic infant prophylaxis or suboptimal maternal ART ingested during breastfeeding. Adopting these strategies should further reduce mother-to-child HIV transmission.

PART III: AFFORDABLE DIAGNOSTICS FOR IMPROVED ART MONITORING

In this section, we assessed affordable diagnostics to improve treatment monitoring in LMICs. In **Chapter 12**, we describe the challenge imposed by the WHO recommendations to use stringent virological failure thresholds for dried blood spots (DBS)-based assays.[25] Although DBS offers a practical solution to the challenge for transporting samples from remote areas without the need for cold-chain systems, their performance with most of the current viral-load (VL) assays is sub-optimal.[26] VL testing based on DBS gives low amplification sensitivity (due to small sample volume) and a lower specificity (contribution by cell-associated viruses in blood which may overestimate the VL).[26] Previous WHO guidelines had tried to account for this by recommending use of higher threshold (≥3000-5000 cps/ml, 2013)[27] but this was since revised in 2014 (≥1000cps/ml).[28] Our analysis showed that using more stringent VL thresholds with DBS-based assays, in fact, might increase programmatic costs associated with unnecessary switches to second-line treatment and additional confirmatory tests that are required. This is important in light of the marginal number of patients (~3.9%) failing treatment who may be missed when using the less stringent thresholds. Overall, these findings illustrate the dilemma that programs and researchers have in trying

to provide ideal versus practical solutions for improving access to better ART monitoring diagnostics in LMICs.

In **Chapter 13** we evaluated the performance of a low-cost, DBS-based in-house Sanger-based genotypic assay that can potentially be used to increase access to HIV drug resistance testing in sub-Saharan Africa.[29] Overall, this assay had a good accuracy (amplification success rate, 89%: sensitivity and specificity to detect DRMs 97% and 100% respectively) compared with plasma-based FDA approved ViroSeq assay and had potential to reduce the costs by up to 60%. Since then, the assay has been adopted into a commercial kit-based test (ABI HIV-1 genotyping kit, formally ATCC) and made available for use in PEPFAR supported countries at a cost of about 50 USD /test. The assay has been adopted by about 20 CDC and World Health Organization laboratories in Africa and Asia. Research is also ongoing to incorporate genotyping of the integrase gene in preparation for the wide-scale roll out of INSTI-based regimens in LMICs.

In **Chapters 14 and 15**, we further reviewed the existing landscape of HIVDR technologies to identify low-cost assays and operational aspects needed to expand access to resistance testing in resource-limited settings.[30,31] Potential affordable technologies include: (i) in-house Sanger-based assays; these are currently considered to be the gold standard genotyping technology and are available at a limited number of LMICs reference laboratories. High capital and test cost have however limited their wide expansion; (ii) point mutation assays; these present opportunities for simplified laboratory assays, but HIV genetic variability, extensive codon redundancy at or near the mutation target sites with limited multiplexing capability have restricted their utility; (iii) next-generation sequencing; these have potential to reduce the testing cost significantly through multiplexing in high-through put facilities, but are unideal for use in low-throughput facilities; (iv) web-based genotype-free prediction systems; these provide enhanced ART decision-making without the need for laboratory testing, but requires further clinical field evaluation and implementation science research in LMICs. Overall, the existing diagnostic landscape shows promise, but implementation research, adaptation of existing normative guidance and political commitment is still needed to support appropriate investments and policy changes.

Although a technological revolution has seen a decline in the cost of sequencing using next-generation technologies, their implementation is hampered by the lack of consensus on the clinical relevance of minority variants.[32,33] In **Chapter 16**, we determined the resistance detection thresholds needed to operationalize the use of these assays. Using a case-control approach involving 399 patients within the PASER-M cohort, we evaluated the optimum resistance detection threshold that predicted risk of virological failure at 12 months on an NNRTI-based first-line regimen. We calculated diagnostic accuracy measures and assessed odds of virological failure using logistic regression for 1%, 5%, and 10% PDR detection thresholds, compared with ≥20% in Sanger sequencing. Lowering the threshold from 20% to 10%, 5% and 1% resulted in improved sensitivity (ability to identify cases) from 12% to 13%, 15% and 17%, respectively, at a cost of reduced specificity (ability to identify controls) from 98% to 96%, 96% and 92%, respectively, and yielded diagnostic odds ratios of 5.4, 3.8, 3.8 and 2.3, respectively. The presence of PDR increased the odds of virological failure by 9.2, 6.8, 7.6 and 4.5, respectively. Our findings showed that incorporating minority variants may improve the prognostic value of HIVDR tests, whereas very low (1%) thresholds compromise test specificity. Further modeling studies are needed to estimate the optimal trade-off and project overall implications for the different thresholds.

FUTURE DIRECTIONS: HOW TO REACH THE THIRD "90" GOAL?

Global efforts for HIV/AIDS have now been galvanized around achieving the UNAIDS 90-90-90 targets designed as milestones on the path to AIDS elimination, and projected to translate to 73% of all people living with HIV/AIDS being on ART and virally suppressed by 2020.[3] Achieving high ART coverage and viral suppression is expected to not only reduce mortality and illnesses but also improve quality of life of HIV infected individuals and eliminate HIV transmission at the population level.

However with only two years left in the period for achieving the 90:90:90 goals, an estimated 15.2 million people living with HIV are yet to receive ART and the overall suppression rate is estimated at only 47% against the 73% 2020 target.[1]

Potential measures that may be taken to aid the achievement of the third 90' target include the following:

1. Timely implementation of ART guidelines to respond to high levels of PDR

The 2017 WHO HIVDR global report showed that in 6 of 11 countries surveyed in LMICs, over 1 in 10 people starting ART had virus that was already resistant to NNRTI-based therapy.[5] PDR to NNRTIs has been associated with poor virological outcomes, impaired immune recovery, reduced durability of NNRTI-based regimens, and increased mortality. [6,34–37] Delay in responding to the rising levels of NNRTI PDR is projected to fuel an increase in mortality, HIV incidence, and antiretroviral therapy (ART) costs. [35] It is thus imperative for countries to rapidly respond to high levels of drug resistance so as to minimize the associated negative public health impact.

In 2017, WHO recommended the use of a non-NNRTI based regimen in countries with PDR ≥10% as well as in all people with prior ARV exposure.[6] Updated guidelines in 2018 reinforced these recommendations by supporting the use of DTG as the preferred first-line ART due to its superior efficacy, tolerability and availability of a low-cost, fixed-dose generic formulation.[38] Transition to DTG-based regimen is forecasted to result in 16% increase in the proportion of patients with viral-suppression a year after ART initiation, reduction in annual mortality of 1 death per 100 people on ART, and a 10% annual reduction in HIV incidence.[39]

By June 2018, five of the six countries that reported PDR levels exceeding 10% were in the process of adapting their guidelines to transition to a DTG-based first-line regimen.[40] However the transition plans have been delayed following safety concerns for the risk of neural tube defects in infants born to women who are on DTG at the time of conception. [41] Due to this, WHO recommended the use of DTG in women only when an effective and reliable contraceptive can be assured.[38] Following this, some countries are exercising caution in using DTG due to limited access to contraceptives, with some like Kenya recommending its use only in men.[42] In sub-Saharan Africa, women comprise 60-70% of people living with HIV and access to contraceptives is limited (36% in 2017).[43] The 2017 WHO HIVDR report also showed that PDR was two times higher in women compared to men suggesting the need for an alternative regimen for this group.[5]

In countries where PDR levels are ≥10% and use of reliable contraceptives cannot be assured, WHO recommends using ritonavir-boosted atazanavir (ATV-r) in women of childbearing potential, pending evidence from ongoing studies.[38]

However, the cost for ATV-r is nearly three times that of DTG or efavirenz (EFV)-based regimen and may present new challenges to most ART programs. [44] The use of efavirenz with close treatment monitoring and switch to PI-based second-line ART upon treatment failure could be a cost-effective strategy, where adequate systems for routine viral load monitoring exists.

2. Addressing alarming rates of PDR in children

In this thesis, we highlighted the worrisome high prevalence of PDR in a particular risk group: children. In an era of limited pediatric HIV treatment options, there is a need for the HIV response in children to also focus on PDR prevention. Treatment outcomes in children are generally poor, attributed mainly to high levels of PDR and lack of more potent regimens[45,46]. The decreasing population of HIV-infected children (due to the success of PMTCT) is not stimulating the development of new formulations by the pharma industry. In response, WHO has since 2013 made an effort to bring together cross-sectoral collaborations which includes manufacturers, research networks, funding bodies, supply and procurement organizations, policymakers and regulatory agencies with the aim of ensuring accelerated development and uptake of optimal pediatric ARVs.[47] However, much still needs to be done, citing the experience with lopinavir-ritonavir pellets which are still inaccessible to most countries due to limited production and high prices.[24] Preventing PDR in infants could be a complementary strategy. There is sufficient knowledge and necessary tools to prevent PDR and increase the armamentarium of pediatric ART needed to support lifelong therapy, including: (i) increasing the frequency of viral load monitoring and using this as an intervention to also assess and prevent the risk of mother-to-child transmission of HIV; (ii) Using INSTI based regimen to accelerate rapid viral suppression during pregnancy and post-partum; (iii) In addition, standard triple ART prophylaxis (ZDV/NVP/3TC) which has a higher genetic barrier for resistance than the current NVP mono- or NVP-ZDV dual-infant prophylaxis, is a more practical solution for prevention of PDR in these settings as it is safer and available in pediatric friendly formulation. Furthermore, raltegravir based ART (raltegravir/abacavir/lamivudine), which has recently been approved for treatment of infants[48], can also be used for prophylaxis due to its better safety profile and higher genetic barrier than NVP based regimen. It is worth noting that despite insufficient evidence, western countries have adopted the use of triple ARV as standard prophylaxis in HIV-exposed infants. Moreover, triple ARV prophylaxis is the preferred post-exposure prophylaxis strategy for both adults and children based on WHO recommendations.[38]

3. Improving access to and functional use of viral load monitoring

Expanding and consolidating universal access to regular viral load monitoring is critical for early identification of virological failure and timely detection of patients with challenges in adherence.[49] A recent review of data from 45 high burden HIV countries showed that in 2017, nearly half of the patients on ART did not receive at least one VL test.[40]There is thus a need for accelerated access.

The current strategy for VL expansion mainly depends on a referral network that uses DBS specimen type to facilitate sample transportation from peripheral sites to centralized high throughput laboratories. This is coupled with global access price deals, bundled agreements and equipment rentals to reduce the cost of laboratory-based VL instruments and reagents.

Since 2014, efforts by UNAIDS, UNITAID, and partners under the Diagnostics Access Initiative, has seen the development of point-of-care tests which are being fronted as alternative strategies to improve VL-test access in remote settings as well as reduce the turn-around-time of test results for clinical decision making.

Although a combination of laboratory-based testing and point-of-care tests may help improve testing access, more still needs to be done to ensure universal access and efficient use of available capacity. Studies indicate underutilization of existing capacities[50] and lack of or delayed action on the test results.[51,52] Recent review of programmatic data in Kenya showed that only 4.1% of patients with unsuppressed viral load received a confirmatory viral load test,[51] while another study in Uganda showed that only 66% of patients with confirmed virological failure were switched to second-line ART. The time to switching after detection of VL failure was much longer estimated at a median time of 8 months.[53]

This calls for addressing the gaps in the viral load cascade, including creating demand by increasing treatment literacy among communities, use of m-health for timely identification of patients in need of viral-load tests, mapping equipment based on need within the catchment area, and timely relay and use of test results. Several initiatives are already ongoing to assist with this and need to be expanded. This includes the MSF access campaign, which addresses both programmatic, and laboratory challenges across the viral load cascade,[54] ASLM training & mentorship program for sharing best practices for viral load scale-up and laboratory system strengthening between ART programs and other

stakeholders,[55] WHO laboratory quality improvement program (WHO African region Stepwise Laboratory Improvement Process Towards Accreditation (SLIPTA) programme),[56] efforts by Global Funds, PEPFAR and WHO to guide on improving viral load testing efficiency among others.

4. Role of drug resistance and therapeutic drug monitoring tests

The current transition to use ARVs with a high-genetic barrier like DTG or PIs in first- line may trigger a shift to using HIVDR tests for individual treatment monitoring in LMICs as empiric switches could be premature citing the rare occurrence of resistance in viremic patients on these regimens.[57,58] This may help prevent unnecessary switches to more expensive or less-tolerated regimens and improve the durability of patients on the limited available drugs. Several countries in sub-Saharan Africa like Botswana,[59] Kenya,[60] Uganda[61] and South Africa[62] have already adopted individualized resistance testing for second-line failures.

To support such endeavors more efforts are needed to reduce the costs of HIVDR tests and increase capacity (technical and infrastructure) in LMICs. In the past years, financial support from multi-national organizations under the Diagnostics Access Initiatives have accelerated the development of low-cost CD4 and viral-load technologies, including point-of-care devices.[63] Similar support should be extended to innovators and manufacturers of HIVDR technologies. This could lead to more innovative technologies that combine VL and HIVDR testing. In addition, collective negotiations by international entities, like CHAI or UNITAID, could further lower the overall costs of both the technologies and the tests.

Coupled with this, is the need for more innovative low-cost HIVDR methodologies for population-based surveillance. Today the annual costs of comprehensive HIVDR surveillance are estimated by WHO at $227,000 per year[64], which can be judged prohibitive by many LMICs. Potential complementary approaches some of which are also recommended by WHO include:

- Optimized surveillance in population most-at-risk of developing drug resistance e.g. using evidence from routinely collected viral-load data, to conduct acquired drug resistance (ADR) survey in groups with high virological non-response. This, however, could be limited only to areas with high VL testing coverage.
- Leveraging on routinely collected programmatic data to conduct ADR surveys using remnant viral-load specimens from patients experiencing

virological failure. WHO is already developing survey methods to guide countries on the use of this approach.[40]

- Continuous monitoring of program quality indicators (early warning indicators, EWIs) associated with the emergence of preventable HIV drug resistance as recommended by WHO.[64] This includes clinic level monitoring of on-time pill pick up, retention to care, drug stock-outs, viral load testing completion, and suppression rates and appropriate and timely switch to second-line ART. Currently WHO recommends the integration of EWI monitoring into routine monitoring and evaluation systems to facilitate real-time assessment and response to sub-optimal program functioning.[65]
- Big data analyses, involving non-sequencing data such as the EWIs and other (un) related databases (like ART coverage, viral load test availability, HIV prevalence and incidence, socio-economic survey data, etc.) to predict HIVDR hotspots.

Apart from EWIs, these proposed approaches will require proof-of-principle, validation and scaling strategies before wide adaptation in LMICs.

Therapeutic drug monitoring may also aid in identifying patients with sub-optimal adherence as well as assess the effectiveness of enhanced adherence intervention. This ideally should include easy-to-use point of care tests. Initial results from a proof of concept study involving a laboratory-based urine tenofovir drug-concentration assay are promising and may potentially serve as an impetus for the development of urine-based drug concentration measurement assays for other ARVs as well as the adaptation of these assays into point-of-care tests. [66]

5. Provision of better, more effective treatment and enhancing research into functional cure strategies

Even with the development of improved diagnostics, the need for better and safer drugs will remain. The recent recommendations to use DTG in first and second-line ART is likely to improve treatment outcomes in LMICs but as previously discussed its wide-scale use in women could be limited by the safety concerns for birth defects in infants. An equivalent potent drug for use in this population could be DRV-r based regimen, but its use is prohibited by high costs. Increased efforts to avail DRV-r at affordable costs in LMICs are needed, noting that it's also recommended for use in second- and third-line ART in these settings.

The ARV landscape is rapidly changing to include drugs from newer classes such as capsid inhibitors and CD4 attachment inhibitors with initial studies showing promising results.[67,68] As with DTG, more efforts may be required to avail these drugs at affordable prices and in a timely manner in LMICs once proven safe and efficacious.

Ongoing research on long-acting formulations of existing and new ARVs also seems promising and may help relieve the burden of adherence to daily medications in specific groups and situations.[69] Efforts will however be needed to overcome the barriers for wide implementation of these formulations in LMICs. These include enabling timely manufacture of low-cost generic drugs, simplified delivery approaches, assessing patient preferences and effective laboratory monitoring.

A more lasting solution for addressing the challenges of adherence may require renewed focus and acceleration in functional cure research (sustaining virological control in absence of treatment) and therapeutic vaccines (enhancing host-mediated anti-HIV immunity to allow HIV control in absence of treatment.

6. Personalizing ART care

Another approach to enhance the quality of ART service delivery and reaching the 'third 90' is to tailor services according to individual needs, i.e. need-based care. To facilitate this, differentiated patient-centered care models are being implemented with the aim of improving adherence, retention in care, overall treatment outcomes and reducing costs and burden to the health care systems.[70,71]

Of particular importance is the need to use such approaches to improve treatment outcomes in adolescents. Notably, adolescents have the highest rates of poor adherence, treatment failure and HIV related deaths as compared to all other age groups.[14–16] A patient-centered model for adolescents may include leveraging on digital-adherence mobile applications interventions, with a focus on peer-peer support, skill building, help-desks with clinicians and personalized reminders.[72]

7. Creating systems for sustainable financing

Achieving and sustaining the projected UNAIDS targets will require a paradigm shift in financing citing the current donor fatigue. Although domestic funding has been increasing, it has generally not been able to match external funding or fill the existing financial gaps. In a recent report, domestic funding contribution

for HIV response in sub-Saharan Africa countries was estimated at only 31% (range 0.8-73.1%).[73]

Even though external aid is unsustainable, relying entirely on domestic funding may be impractical and equally unsustainable due to the high costs of HIV/AIDS programs amounting to between 50% and two-thirds of the entire health budget allocations in these countries.[74] With other competing health needs, including other hyper-endemic infectious diseases and a rising burden of non-communicable diseases, it may not be possible for most low-income countries to commit such huge expenditures to the HIV response.

This calls for the need for innovative financing approaches for both domestic and external funding. Examples of existing approaches include incorporating HIV/AIDS services as part of universal health coverage schemes or other forms of insurance schemes as is the case with Brazil, Thailand, and other middle-income countries.[74] Special levies dedicated to HIV response as well as international debt conversion for health have also led to improved domestic funding in some countries.[75] The current global goal to achieve universal health coverage by 2030 has also steered an increased focus for pooling resources for health, which may likely be a more feasible way to fund HIV programs.

Similarly, novel approaches are needed to mobilize global funds for HIV. The deficit in the current HIV financing is estimated at 7.2 billion USD and is expected to rise as more patients are initiated on treatment.[76] This is amidst severe budgetary constraints by donor countries, which have led to reduced funding towards HIV response. Financing from solidarity levies from global taxes, and other innovative ways like "product red" (unique red-branded product and services from iconic companies whose profits are shared with Global funds) have been quite successful and need to be enhanced.[77] Nonetheless, there's still a need for alternative financing approaches such as crowd funding.

Beyond financing, sustainability will also depend on sustaining the political will, community and advocacy support, support of the evidence-based strategies used by donors including effective supply-chain systems, data, monitoring and evaluation systems among others. Sustaining the HIV response will also mostly rely on an integrated primary health care model as opposed to vertical, parallel system characteristic of the donor-funded programs. There will however be a need to ensure high-quality services in the integrated systems.

There will also be a need to sustain key structures in the global community that will ensure the continuous supply of affordable drugs and laboratory test commodities, oversight and policing the ART programs and ensuring rapid responses in case of instabilities and epidemic resurgence.

Beyond attainment of the 3rd 90

There are doubts about whether achieving the UNAIDS targets will indeed lead to epidemic control. Recent findings from countries like Botswana, Eswatini and Namibia, that have nearly achieved these targets reveal only modest reduction in incidence or mortality.[1,78] As more countries achieve the 2020 90-90-90 targets, there is need for a comprehensive audit on the effectiveness and efficiency of the current intervention strategies for epidemic control. Over-reliance only on ART may eclipse the myriad complex factors that have in the past made it difficult to achieve sustained viral suppression or prevent new HIV infections. Achieving epidemic control will require a more comprehensive approach that incorporates all available tools. Of importance, is the need for renewed focus and acceleration of research in functional cure (sustaining virological control in absence of treatment) as well as preventive vaccines, which are strategies that are likely to ensure a sustainable long-term control of the HIV epidemic.

CONCLUSION

Looking ahead, there is hope that with increased efforts, the UNAIDS targets for having 73% viral suppression rate in all HIV infected patients may be achieved by 2020. The findings in this thesis highlight potential factors that may hinder the attainment of this goal. Timely response to the rising level of drug resistance in accordance with WHO recommendations and provision of more efficacious and well-tolerated ART regimens will be critical in improving treatment outcomes. Adoption of person-centred care approaches especially among adolescents will be vital to improve adherence and retention. Ensuring wide-scale access to routine viral load and where possible drug resistance testing will improve ART monitoring and help inform timely and appropriate switching to the next line of treatment. Efforts to attain wide-scale sustained viral load suppression should also incorporate research in long-acting ARVs and functional cure, which are likely to effectively address the challenges of long-term poor ART adherence.

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ADDENDUM

Nederlandse samenvatting (summary in dutch)

In de laatste vijftien jaar is de beschikbaarheid van antiretrovirale therapie (ART) voor mensen die geïnfecteerd zijn met hiv in sub-Sahara Afrika sterk verbeterd; in 2017 had ongeveer 60% van de 25.7 miljoen toegang tot ART. Dit heeft geresulteerd in sterke verlaging van hiv-gerelateerde morbiditeit, sterfte en incidentie; in 2017 was het aantal jaarlijkse hiv-gerelateerde doden met bijna 30% verminderd ten opzichte van 10 jaar eerder .

Met de verbeterde toegang tot ART, verplaatst de aandacht zich in toenemende mate van het voorkomen van aids en dood, naar het streven naar goede gezondheid op de lange termijn, verbeterde kwaliteit van leven en het voorkomen van nieuwe infecties. UNAIDS heeft in 2014 de volgende doelen geformuleerd, namelijk dat 90% van mensen met hiv ook gediagnosticeerd zijn, dat 90% hiervan ART krijgt en dat 90% hiervan volledige onderdrukking heeft van de virusreplicatie. Met het behalen van deze doelen is daarna het uiteindelijke doel het onder controle krijgen van de hiv-epidemie in 2030.

Er zijn meerdere factoren die het bereiken van deze doelen bemoeilijken. Dit proefschrift concentreert zich op het probleem van het behalen van de derde "90", namelijk virale onderdrukking. De studies in dit proefschrift zijn onderdeel van de "Pan-African Studies to Evaluate Resistance" (PASER-M), een multicentrisch cohort in zes Afrikaanse landen; Kenia, Nigeria, Zuid-Afrika, Uganda, Zambia en Zimbabwe. De beschreven studies in dit proefschrift gaan over het identificeren van determinanten van langdurige succesvolle virale onderdrukking door ART, het beschrijven van therapieresistentie in kinderen en het evalueren van nieuwe betaalbare diagnostische testen voor betere ART monitoring.

DEEL I: DETERMINANTEN VAN LANGDURIGE ONDERDRUKKING VAN DE VIRALE REPLICATIE DOOR ART

In Deel I evalueren we factoren die langdurige virale onderdrukking beïnvloeden. In **Hoofdstuk 2**, evalueren we het effect van eerder gebruik van antiretrovirale middelen (ARV) (als behandeling of profylaxe) op virale respons na (her)starten van ART en in hoeverre dit effect word veroorzaakt door therapieresistentie. De

WHO schat dat 1 op de 5 patiënten die eerstelijnsbehandeling in Sub-Sahara-Afrika starten eerder ARVs heeft gebruikt. In het PASER-M cohort hadden mensen met eerder ARV-gebruik een 7x verhoogde kans op therapieresisentie en een 3x verhoogde kans op virologisch therapiefalen in vergelijking met ARV-naïeve patiënten. Deze bevindingen benadrukken de noodzaak voor het gebruik van geïndividualiseerde eerstelijns ART voor patiënten met eerder ARV-gebruik. Op basis van deze en andere onderzoeken, heeft de WHO in 2017 nieuwe richtlijn uitgegevenmet daarin het advies om als eerstelijns behandeling voor patiënten die eerder ARVs hebben gebruikt geen non-nucleoside reverse transcriptase inhibitor (NNRTI) te gebruiken.

In **Hoofdstuk 3**, evalueren we de impact van PDR op verschillende NNRTI eerstelijnsbehandeling in het PASER-M cohort. Deelnemers die PDR hadden en niet-tenofovir+lamivudine/emtricitabine kregen met efavirenz of nevirapine, hadden een verhoogd risico op virologisch falen, in vergelijking met patiënten zonder PDR. Dit risico was echter alleen van borderline-significantie voor deelnemers die een tenofovir-bevattend regime kregen. Deze bevindingen suggereren een lagere impact van PDR op eerstelijns behandeling met tenofovir vergeleken met een thymidine analoog.

In **Hoofdstuk 4** hebben we de duurzaamheid van eerstelijnsbehandeling in een cohort van 955 patiënten in West-Kenia onderzocht. Binnen een periode van gemiddeld 10,7 maanden ART, was bij 18,7% van de patiënten de ART-behandeling aangepast, voornamelijk als gevolg van bijwerkingen op de gebruikte middelen. Het gebruik van tenofovir (TDF) en zidovudine (ZDV) zorgde voor een vermindering van 49% en 40% van het risico op het veranderen van behandeling, ten opzichte van stavudine (d4T). Deze resultaten waren niet verrassend, omdat het al lang bekend is dat d4T veel bijwerkingen en toxiciteit met zich mee brengt. De ervaring met d4T benadrukt de noodzaak van een betere geneesmiddelenbewaking, brede toegang en snelle overgang naar betere en beter verdraagbare medicijnen om de duurzaamheid van de beperkte beschikbare behandelopties in Afrika te vergroten.

Optimale therapietrouw is essentieel om langdurige virale suppressie te bereiken. In **hoofdstuk 5** hebben we de bevindingen beschreven van een kwalitatief onderzoek met behulp van diepte-interviews met zorgverleners om de determinanten van langdurige ART therapietrouw bij adolescenten

en volwassenen in Oeganda te beoordelen. Adolescenten zijn een bijzonder uitdagende groep met slechtere behandelingsuitkomsten dan volwassenen of kinderen. Slechte therapietrouw bij adolescenten werd veroorzaakt door ongeplande onderbrekingen, laat openbaren van de hiv-diagnose door verzorgenden, stigma (vooral op kostscholen), gebrek aan ondersteuning voor jonge volwassenen tijdens de overgang naar volwassenenzorg, en onvoldoende lotgenotenondersteuning. Belangrijke belemmeringen voor therapietrouw bij volwassenen waren: gebrek aan openheid over hiv in intieme relaties, medicatiebijwerkingen, gebruik van afzonderlijke tabletten in plaats van vaste-dosis-combinatie pillen, onaangename smaak en onvoldoende begeleiding door professionals. Deze bevindingen wijzen op de noodzaak van specifieke interventies afgestemd op langdurig behandelde patiënten.

In **hoofdstuk 6** beschrijven we patiënten die therapiefalen ontwikkelen op tweedelijnsbehandeling in Kenia. In deze nationale studie had 1 op de 4 patiënten die faalden op tweedelijns-ART geen alternatieve beschikbare behandelopties. Deze studie onderstreept de noodzaak van prijsreducties en verbeterde toegang tot derdelijnsbehandeling in Kenia.

In **Hoofdstuk 7** hebben we de primaire resistentie tegen de ARV-klasse van de integraseremmers (INSTIs) onderzicht in alle belangrijke hiv1-subtypen die circuleren in sub-Sahara Afrika. Van de 425 virale isolaten in het PASER-M-cohort, was 49% subtype C, 29% A, 10% D, 3% G en 10% waren recombinanten. "Major" resistentiemutaties waren zeldzaam en kwamen alleen voor in lage concentraties;. De bevindingen wijzem erop dat INSTIs waarschijnlijk effectief zijn in de verschillende subtypes die circuleren in sub-Sahara Afrika.

Hoofdstuk 8 is een opiniestuk over het gebruik van de INSTI dolutegravir (DTG) als mogelijke oplossing voor hiv therapieresistentie in Afrika en het gebrek aan gegevens. Er is meer aandacht nodig om de structurele factoren die resistentie in de hand werken aan te pakken, opdat het gebruik van DTG in ontwikkelingslanden inderdaad kan resulteren in een meer effectieve en duurzame behandeling. Belangrijke vragen zijn nog onbeantwoord, zoals: hoe effectief DTG is als het gecombineerd wordt met 2 nucleoside reverse transcriptase remmers (NRTIs) waartegen mogelijk al resistentie bestaat? Wat zijn de genetische mechanismen van resistentie tegen DTG in non-B hiv-1 subtypes? Wat de beste manier is om patiënten die DTG gebruiken te monitoren met laboratoriumtesten? Bovendien

blijkt DTG mogelijk onvoldoende veilig voor vrouwen in de vruchtbare leeftijd, waardoor er een andere oplossing voor therapieresistentie nodig is voor deze groep.

DEEL II: PRE-THERAPIE RESISTENTIE (PDR) BIJ HIV-GEÏNFECTEERDE KINDEREN

De succeskansen van ART zijn voor kinderen in Afrika over het algemeen lager dan voor volwassenen. Dit komt omdat kinderen minder behandelopties hebben, minder toegang tot ART en dat de risico's op therapieresistentie groter zijn als gevolg van blootstelling aan ARV-profylaxe ter voorkoming van moeder-kind transmissie (PMTCT). In Deel II hebben we de PDR bij kinderen en mogelijke oplossingen onderzocht.

In **Hoofdstuk 9** beschrijven we K65R, een multi-NRTI-resistentiemutatie, bij kinderen die borstvoeding krijgen en worden blootgesteld aan ARV-profylaxe van de moeder in Kisumu, Kenia. De bevindingen suggereerden dat de K65R-mutatie werd verkregen door blootstelling aan suboptimale ARV-profylaxe van de moeder.

In **Hoofdstuk 10** beschrijven we de hoge prevalentie van PDR bij zuigelingen (<18 maanden) in een nationaal onderzoek in Nigeria. In totaal had 48% van 430 baby's PDR, voornamelijk tegen NNRTIs (45%) en 20% had resistentie tegen zowel NNRTIs als NRTIs. De prevalentie van PDR bij kinderen met blootstelling aan PMTCT was 57% en bij de niet-blootgestelde baby's 34%. Op basis van deze gegevens is eerstelijnsbehandeling met een proteaseremmer (PI) aanbevolen voor alle baby's, ongeacht blootgestelling aan PMTCT.

Hoofdstuk 11 is een opiniestuk over mogelijke strategieën om PDR bij baby's te voorkomen. De WHO adviseert PIs als eerstelijnsbehandeling, maar in de praktijk zijn de kosten te hoog en zijn er logistieke beperkingen. Hierdoor krijgt tot 76% van de zuigelingen nog steeds ART-regimes gebaseerd op NNRTIs, ondanks het verhoogde risico op therapiefalen. Het voorkomen van de opkomst van PDR is essentieel, gezien de beperkte behandelopties voor kinderen. Beter gebruik van virale load metingen bij de moeder voor de bevalling en tijdens de borstvoeding kan hiv-overdracht naar de baby voorkomen. Interventies gericht op bevorderen

van de therapietrouw en het gebruik van INSTIs voor degene die falen op therapie bijdragen aan het voorkomen van HIVDR overdracht. ARV-profylaxe bij kinderen bestaande uit triple-ART of een regime met een hoge genetische barrière voor resistentie kan PDR voorkomen.

DEEL III: BETAALBARE MOLECULAIRE DIAGNOSTIEK VOOR VERBETERDE ART-MONITORING

In dit deel hebben we betaalbare, praktische diagnostische testen geëvalueerd om het controleren van de effectiviteit van ART-behandeling in ontwikkelingslanden te verbeteren. In **hoofdstuk 12** beschrijven we de uitdaging van de WHO-aanbevelingen om strenge criteria voor virologisch falen te gebruiken voor tests op basis van "dried blood spot" (DBS). VL-testen op basis van DBS geven een lage amplificatie sensitiviteit (vanwege een klein monstervolume) en een lagere specificiteit (bijdrage door ander virussen in het bloed die de VL kunnen overschatten). Eerdere WHO-richtlijnen hebben dit geprobeerd op te lossen door een hogere drempel aan te bevelen (≥ 3000-5000 cps/ml) maar dit werd in 2014 herzien (≥1000cps/ml). Onze analyse suggereert dat het gebruik van strengere VL-drempels met op DBS gebaseerde testen de kosten zou kunnen verhogen, omdat er onnodig vaak overgestapt wordt naar tweedelijnsbehandeling.

In **Hoofdstuk 13** hebben we de prestaties geëvalueerd van een op DBS gebaseerde in-house genotypering test die kan worden gebruikt om de toegang tot hiv-resistentietesten in Sub-Sahara Afrika te verbeteren. Over het algemeen had deze assay een goede nauwkeurigheid vergeleken met de op plasma gebaseerde door de FDA goedgekeurde ViroSeq-test en had potentie om de kosten tot 60% te verlagen. Sindsdien is de test beschikbaar gemaakt als commerciële kit voor een prijs van ongeveer 50 USD per test. De test is door ongeveer 20 CDC- en WHO-laboratoria in Afrika en Azië goedgekeurd.

In de **hoofdstukken 14 en 15** hebben we de bestaande technologieën verder onderzocht om goedkope testen en operationele aspecten te identificeren die nodig zijn om de toegang tot resistentietests uit te breiden in ontwikkelingslanden. Potentieel betaalbare technologieën zijn:(i) in-house Sanger-gebaseerde testen; (ii) puntmutatie-assays; (iii) next-generation sequencing; (iv) predictiemodellen zonder genotypering (op basis van Big Data). Al met al zijn de nieuwe diagnostische

HIVDR technologieën veelbelovend, maar implementatieonderzoek, aanpassing van bestaande richtlijnen en politieke wil zijn nodig om passende investeringen en beleidswijzigingen te realiseren.

Hoewel de kosten van sequencen met behulp van next-generation technologieën zijn gedaald, wordt de implementatie gehinderd door het gebrek aan consensus over de klinische relevantie van hiv-varianten die in lage frequentie voorkomen. In **hoofdstuk 16** hebben we de detectiedrempels van resistente virsuvarianten bepaald om het gebruik van deze assays te operationaliseren. Aan de hand van een case-control studie met 399 patiënten binnen het PASER-M-cohort evalueerden we de optimale resistentie detectiedrempel die het risico op virologisch falen voorspelde na 12 maanden op basis van een op NNRTI-gebaseerde eerstelijnsbehandeling. Onze bevindingen lieten zien dat het meten van varianten die in lage frequentie voorkomen de prognostische waarde van HIVDR-testen kan verbeteren, hoewel het gebruik van een zeer lage (1%) drempel de testspecificiteit doet verslechteren. Verdere modellering studies zijn nodig om een optimale drempel te bepalen wat betreft klinische beslissingen en kostimplicaties.

RESUMEN (SUMMARY IN SPANISH)

Los esfuerzos mundiales con respecto al VIH/SIDA se han visto redoblados con el fin de alcanzar los objetivos 90-90-90 propuestos por ONUSIDA (que el 90% de las personas que viven con VIH conozcan su estado serológico respecto al VIH, que el 90% de los infectados reciba tratamiento antirretroviral (TAR), y que el 90% de quienes reciban TAR tengan supresión viral) que, de alcanzarse en 2020, permitirían el control epidemiológico del VIH en 2030. Múltiples factores suponen un desafío para alcanzar estos objetivos. Esta tesis se centra en el desafío de alcanzar el tercer objetivo '90' sobre la supresión viral, haciendo especial énfasis en la adherencia al TAR, la resistencia del VIH al TAR y la necesidad de un mejor diagnóstico molecular en África subsahariana.

PARTE I: DETERMINANTES DE LA SUPRESIÓN VIRAL SOSTENIDA A LARGO PLAZO

En la Parte I evaluamos los factores que impiden o facilitan alcanzar la supresión viral sostenida a largo plazo. En el **Capítulo 2** evaluamos el riesgo del uso previo de fármacos antirretrovirales (ARVs) con respecto a la respuesta virológica al TAR y la medida en que dicho efecto se derivó de la resistencia pretratamiento del VIH a los ARVs. En este estudio, el 4,5% de los 2.737 participantes que comenzaron TAR de primera línea en la cohorte PASER-M tenían un historial de uso previo de ARVs (incluidos aquellos con exposición previa a ARVs como prevención de la transmisión maternoinfantil del VIH (PTMI) o pacientes que volvían a comenzar TAR tras haberlo abandonado). Esto los predisponía a un riesgo 7,2 veces mayor de tener resistencia pretratamiento del VIH a los ARVs y a un riesgo 3,0 veces mayor de desarrollar un fallo virológico en comparación a los participantes que no habían recibido previamente ARVs. Intervenir en la resistencia pretratamiento del VIH a los ARVs eliminaría el 50% de los efectos del uso previo de ARV sobre el fallo virológico. A partir de las evidencias que arrojaron este y otros estudios, la Organización Mundial de la Salud (OMS) publicó una guía provisional en 2017 recomendando el uso de una primera línea de TAR no basada en los inhibidores no nucleosídicos de la transcriptasa inversa (INNTI) para pacientes con un historial de uso previo de ARVs.

En el **Capítulo 3** evaluamos el impacto de la resistencia pretratamiento del VIH a los ARVs en diferentes esquemas de TAR de primera línea basados en

INNTI en la cohorte PASER-M. Los participantes que presentaban resistencia pretratamiento del VIH a los ARVs y habían recibido TAR basado en efavirenz o nevirapina, emtricitabina/lamivudina y otro inhibidor nucleosídico de la transcriptasa inversa (IN(t)TI) diferente a tenofovir, presentaban un mayor riesgo de fallo virológico en comparación con aquellos que no presentaban resistencia pretratamiento del VIH a los ARVs. Sin embargo, este riesgo estaba en el límite de significancia estadística para los participantes que habían recibido un régimen de TAR con tenofovir. Estos hallazgos sugieren que la resistencia pretratamiento del VIH a los ARVs podrían tener un impacto comparativamente menor sobre el TAR de primera línea basado en INNTI que incluya tenofovir que en aquellos que incluyen IN(t)TI análogos de timidina.

En el **Capítulo 4** evaluamos la durabilidad del TAR de primera línea en una cohorte de 955 pacientes en el occidente de Kenia.[7] En un período de seguimiento medio de 10,7 meses, al 18,7% de los pacientes se les había modificado su régimen de TAR, principalmente, por toxicidades medicamentosas. Los factores de riesgo para las modificaciones de TAR fueron: iniciar TAR en un estadio avanzado de la enfermedad (estadios III/IV de la infección del VIH según la clasificación de la OMS), o con recuentos bajos de linfocitos T CD4+, en edades avanzadas, uso de estavudina (d4T) y el sobrepeso. El uso de tenofovir (TDF) y zidovudina (ZDV) se asoció con unas reducciones del 49% y 40% del riesgo de modificación del tratamiento. Estos hallazgos resaltan la necesidad de una mayor farmacovigilancia, de un acceso más amplio y de una rápida transición a medicamentos mejores y más tolerables para aumentar la durabilidad de las limitadas opciones de TAR disponibles en África.

La capacidad de alcanzar una supresión viral sostenida depende del cumplimiento óptimo de la adherencia al TAR a lo largo de la vida del paciente. En el **Capítulo 5** describimos los hallazgos de un estudio cualitativo que empleaba entrevistas a profundidad con proveedores de salud para evaluar los determinantes de la adherencia al TAR en adolescentes y adultos en Uganda. Los determinantes de una baja adherencia al TAR en adolescentes incluyeron recesos o pausas no estructurados en el uso del TAR, retardo en la revelación del estado serológico respecto del VIH por parte de los proveedores de salud, la estigmatización, la reducción o falta de apoyo clínico para pacientes jóvenes durante la transición a los servicios para pacientes adultos y el cada vez menor apoyo para las actividades entre pares. Los problemas para acceder al tratamiento que sufren

los migrantes estacionales por razones económicas resultaron ser la principal barrera contra la adherencia al TAR en adultos. Barreras frecuentes para la adherencia a TAR en adultos y adolescentes fueron los problemas de revelación del diagnóstico de VIH con parejas sexuales, los efectos secundarios de los ARVs, la provisión de TAR como tabletas individuales en lugar de dosis fijas combinadas de ARVs, el suministro de marcas de medicamentos con mal sabor y la falta de oportunidades de asesoramiento debido a insuficiente personal de salud. Estos hallazgos indican la necesidad de intervenciones personalizadas de adherencia al TAR para problemas que vayan surgiendo a lo largo de la vida de los pacientes en TAR y de un mayor apoyo para adolescentes que viven con el VIH.

En el **Capítulo 6** describimos una creciente preocupación por el agotamiento de las opciones de TAR para pacientes que fallan a los esquemas de TAR de segunda línea en Kenia. En este estudio transversal a nivel nacional, 1 de cada 4 pacientes con fallo virológico en esquemas de TAR de segunda línea habían agotado todas las opciones de TAR disponibles, lo cual puso de manifiesto la necesidad de un mejor acceso a ARVs de tercera línea en estos entornos. Los medicamentos de tercera línea alternativos resultan prohibitivos, y suelen costar entre 6 y 14 veces más que los de primera y segunda línea, por lo que quedan fuera del alcance de los programas de TAR en países con ingresos bajos y medios.

En el **Capítulo 7** evaluamos la resistencia primaria a los ARVs inhibidores de la integrasa entre los principales subtipos de VIH-1 en África subsahariana. De los 425 genotipos de aislados virales entre los participantes en la cohorte PASER-M, un 48,7% eran del subtipo C, un 28,5% del A, un 10,1% del D, un 2,8% del G y un 9,9% eran recombinantes. Se observó una baja la frecuencia de mutaciones asociadas a resistencia a inhibidores de la integrasa, lo que sugiere que estos ARVs podrían resultar efectivos contra los diversos subtipos de VIH en esta región.

En el **Capítulo 8** presentamos una serie de argumentos sobre la cautela que debe de tenerse para no presentar al dolutegravir (DTG) como solución general al aumento de la resistencia del VIH a los ARVs en países de ingresos bajos y medios. En primer lugar, la actual preocupación sobre la seguridad del uso de DTG en mujeres en edad fértil podría implicar la necesidad de una solución alternativa a la resistencia del VIH a los ARVs en este grupo poblacional . En segundo lugar, la resistencia preexistente a los IN(t)TI, que se coadministran como parte del TAR con DTG, podría reducir la efectividad del DTG. En tercer lugar, la información

sobre la correlación genética de la resistencia a DTG es limitada, en particular en pacientes infectados con VIH-1 de subtipos diferentes al subtipo B. Por último, será un desafío el manejo clínico de pacientes con fallo virológico en regímenes de tratamiento basados en DTG dada la incertidumbre con respecto a si se ha desarrollado resistencia a DTG, siendo necesario cambiar a otro ARV, o si la mejora en adherencia a TAR es suficiente. En general, hacemos énfasis en la necesidad de abordar estos desafíos para consolidar las ventajas esperadas del uso generalizado de DTG en países con ingresos bajos y medios.

PARTE II: CARGA DE ENFERMEDAD DE LA RESISTENCIA PRETRATAMIENTO DEL VIH A LOS ARVS EN INFANTES

En la Parte II evaluamos carga de enfermedad de la resistencia pretratamiento del VIH a los ARVs en infantes y las potenciales estrategias de mitigación. En el **Capítulo 9** describimos la aparición de K65R, una mutación asociada a la resistencia de varios ARVs de la familia de los medicamentos IN(t)TI, en lactantes expuestos a regímenes de PTMI (ZDV más lamivudina (3TC), o nevirapina (NVP), y nelfinavir) que se desconocía previamente que seleccionaran esta mutación. Incluimos a 24 niños infectados de VIH durante el período de lactancia (de hasta 6 meses posparto) en el ensayo clínico con lactantes en Kisumu, Kenia. En general, 6 de cada 24 (25%) niños desarrollaron la mutación K65R en seis meses. Ninguna de las parturientas y ninguno de los niños presentaban la mutación K65R en el genotipo basal, lo cual sugiere que no fue transmitida, sino que adquirida por exposición a un régimen subóptimo de TAR con 3TC de las madres. Este estudio puso de manifiesto la necesidad de elucidar el mecanismo de selección de la mutación K65R en presencia de niveles subóptimos de 3TC.

En el **Capítulo 10** describimos una elevada prevalencia de resistencia pretratamiento del VIH a los ARVs en infantes <18 meses de edad en un estudio a nivel nacional en Nigeria. En general, un 48% de 430 niños presentaban mutaciones de resistencia a los ARVs, predominantemente para los INNTI (45%), proporción también elevada entre niños no expuestos a PTMI (34%). Estos hallazgos indican una apremiante necesidad de adoptar las recomendaciones de la OMS para el uso de TAR de primera línea basado en inhibidores de la proteasa (PI) en infantes, independientemente de los antecedentes de exposición a PTMI, en países con ingresos bajos y medios.

En el **Capítulo 11**, discutimos las estrategias potenciales para prevenir la aparición de resistencia pretratamiento del VIH a los ARVs en infantes.[22] No obstante la OMS recomienda el uso de TAR de primera línea basados en PI en niños, los costos y aspectos logísticos representan barreras para la adopción de esta recomendación, por lo que hasta un 76% siguen en regímenes de TAR basados en INNTI, a pesar del alto riesgo de tener resistencia pretratamiento del VIH a los INNTI. [24] Las estrategias para prevenir la resistencia pretratamiento del VIH a los ARVs incluyen el uso de regímenes profilácticos con altas barreras genéticas en infantes expuestos al VIH, aumentar la frecuencia de seguimiento de las cargas virales maternas durante el preparto y todo el período de lactancia, complementado con consejería intensificada en adherencia, y el uso de regímenes de TAR basados en inhibidores de integrasa para madres con fallo virológico para facilitar la rápida supresión viral.

PARTE III: ENSAYOS DE LABORATORIO ASEQUIBLES PARA UN MEJOR MONITOREO DE TAR

En esta sección evaluamos ensayos de laboratorio asequibles para mejorar el monitoreo de TAR en países con ingresos bajos y medios. En el **Capítulo 12** describimos el desafío que supone el uso de umbrales de fallo virológico rigurosos para ensayos basados en manchas de sangre seca (*dried blood spots,* DBS) en papel filtro. Aunque el uso de DBS ofrece una solución práctica al desafío que plantea el transporte de las muestras desde zonas remotas sin sistemas de cadena de frío, su rendimiento con la mayoría de los ensayos de carga viral actuales es subóptimo dada la baja sensibilidad de amplificación (debido al pequeño volumen de muestra) y especificidad (genomas virales intracelulares en la sangre que podrían sobrestimar el valor de la carga viral).[26] Nuestro estudio ha demostrado que usar umbrales rigurosos de carga viral (≥1000 copias/ml) aumentaría los costes programáticos, asociados con cambios innecesarios a segunda línea de TAR y pruebas de confirmación, a pesar de detectar una baja proporción (~3,9%) pacientes con fallo terapéutico que no se detectarían al usarse umbrales menos rigurosos de carga viral para fallo virológico (≥3000-5000 copias/ml).

En el **Capítulo 13** evaluamos el desempeño de un ensayo casero de bajo coste para genotipaje de VIH, basado en la técnica Sanger y en el uso de DBS, que podría aumentar el acceso a las pruebas para detectar mutaciones del VIH asociadas a resistencia a los ARVs en países con ingresos bajos y medios.[29] Este ensayo obtuvo una buena exactitud tanto para DBS como para plasma en comparación con el ensayo ViroSeq, aprobado por FDA para uso con plasma, con un potencial de reducción de hasta un 60% de los costes. Este ensayo fue adaptado para su comercialización con un coste reducido de 50 USD/pruebas para países apoyados por el gobierno de Estados Unidos de América a través de su Plan Presidencial de Emergencia para el Alivio del Sida.

En los **Capítulos 14 y 15** revisamos el panorama actual de las tecnologías para detectar resistencia del VIH a los ARVs para identificar los ensayos de bajo coste y los aspectos operacionales necesarios para ampliar el acceso a estas pruebas con recursos limitados.[30,31] En general, el panorama actual es prometedor, pero sigue haciendo falta implementar investigaciones, adaptar las directrices normativas existentes y garantizar el compromiso político para que tengan lugar unas inversiones adecuadas y cambios en las políticas.

En el **Capítulo 16** determinamos los umbrales de detección de la resistencia necesarios para implementar el uso de potenciales ensayos ultrasensibles de bajo coste. Empleando un enfoque de control de casos con 399 pacientes en la cohorte PASER-M, evaluamos el umbral de detección de resistencia óptimo (1%, 5% y 10%, en comparación con el tradicional 20% empleado en secuenciación basada en la técnica Sanger), predictor del riesgo de fallo virológico a los 12 meses en un régimen de TAR de primera línea basado en INNTI. El uso de pruebas de resistencia ultrasensibles para detectar resistencia pretratamiento del VIH mejoró la identificación de personas con riesgo de fallo virológico, sin embargo, provocó una reducción de nuestra capacidad para identificar personas con supresión viral, especialmente en umbrales muy bajos. Se precisa un modelaje en mayor profundidad para estimar las compensaciones óptimas para equilibrar la mejor búsqueda de casos frente a los cambios innecesarios de régimen de TAR.

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PHD PORTFOLIO

Name of PhD student	S.C. Inzaule
PhD Period	September 2014-August 2017
Name PhD supervisors	Prof. dr. T.F Rinke de Wit
	Dr. Denise Naniche
	Dr. Raph L. Hamers

PhD Training	**Year**	**Workload Hours/ECTS**
General courses		
Athena Institute Vrije University, Amsterdam, The Netherlands		
-Inter- and Transdisciplinary Research for Global Health	2014	6.0
Amsterdam UMC of the University of Amsterdam		
AMC world of science	2014	0.7
Scientific writing for research publication	2016	1.5
Entrepreneurship in health and life sciences	2016	1.5
Oral Presentation in English	2016	0.8
Medical Literature: EndNote	2016	0.1
Family Health International		
Research Ethics	2018	3.0
Specific courses		
Amsterdam UMC of the University of Amsterdam Graduate School, Amsterdam, The Netherlands		
Advanced qPCR	2016	0.7
Clinical Epidemiology: Systematic Reviews	2016	0.7
Clinical Epidemiology: Evaluation of Medical Tests	2017	0.9
Clinical Epidemiology: Observational Epidemiology	2017	0.6
Clinical Epidemiology: Randomized Clinical Trials	2016	0.6
Computing in R	2016	0.4
Erasmus University summer programme, Rotterdam, The Netherlands		
Logistic Regression	2016	1.4
Survival Analysis	2016	1.9
Causal Mediation Analysis	2016	1.4
Health Economics	2016	0.7

ISGlobal University of Barcelona		
Qualitative Research methods	2015	3.0
Seminars, workshops and master classes		
Advances in Epidemiologic Analysis, Erasmus university, Rotterdam The Netherlands	2016	0.4
Advances in Genomics Research, Erasmus university, Rotterdam The Netherlands	2016	0.4
TransGlobal Health Annual Meeting	2014-2017	1.5
Presentations		
Scientific conferences		
Poster presentation at the International Workshop on HIV Treatment, Pathogenesis and Prevention Research in Resource-Limited Settings, Yaoundé, Cameroon	2016	1.5
Poster presentation at the International AIDS Society conference, Durban, South Africa	2016	1.5
Poster presentation at the Netherlands Conference on HIV Pathogenesis, Epidemiology, Prevention and Treatment, Amsterdam, The Netherlands	2016	1.5
Oral presentation at the Netherlands Conference on HIV Pathogenesis, Epidemiology, Prevention and Treatment, Amsterdam, The Netherlands	2017	1.5
Oral presentation at the International Workshop on HIV Drug Resistance and Treatment Strategies, Johannesburg, South Africa	2017	1.5
Poster presentation at the Conference on Retroviruses and Opportunistic Infections, Boston, MA, USA	2018	1.5
Other		
Oral presentation at the HIV Drug Resistance Technical Working Group, Abuja, Nigeria	2017	0.6
Oral presentation at CosmoCaixa Barcelona	2017	0.1
Parameters of esteem		
Travel award to International AIDS society conference, Durban, South Africa	2016	
Young Investigator Award, Conference on Retroviruses and Opportunistic Infections	2018	

LIST OF PUBLICATIONS

This Thesis

Inzaule SC, Rinke de Wit TF, Hamers RL "Does Tenofovir-containing First-line Antiretroviral Therapy Mitigate the Impact of Pretreatment Non-nucleoside Reverse Transcriptase Inhibitor Drug Resistance?" Clinical Infectious Diseases 2018, in press

Inzaule SC, Hamers RL, Doherty M, Shafer RW, Bertagnolio S, Rinke de Wit TF "Curbing the impact and rise of HIV drug resistance in low- and middle-income countries: the role of dolutegravir-containing regimens" The Lancet Infectious Diseases 2018, (accepted)

Inzaule SC, Kityo CM, Siwale M, Akanmu AS, Wellington M, de Jager M, Ive P, Mandaliya K, Stevens W, Boender TS, Ondoa P, Sigaloff KCE, Naniche D, Rinke de Wit TF, Hamers RL "Previous antiretroviral drug use compromises standard first-line therapy in Africa and is mediated through drug-resistance" Scientific Reports 2018;8(1):15751

Inzaule SC, Hamers RL, Noguera-Julian M, Casadellà M, Parera M, Kityo C, Steegen K, Naniche D, Clotet B, Rinke de Wit TF, Paredes R, for the PanAfrican Studies to Evaluate Resistance (PASER) "Clinically relevant thresholds for ultrasensitive HIV drug resistance testing: a multi-country nested case-control study" Lancet HIV 2018, 5(11):e638-e646

Inzaule SC, Hamers RL, Noguera-Julian M, Casadella M, Parera M, Rinke de Wit TF and Paredes R "Primary resistance to integrase strand transfer inhibitors in patients infected with diverse HIV-1 subtypes in sub-Saharan Africa" Journal of Antimicrobial Chemotherapy 2018, 73(5):1167-1172

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Inzaule SC, Weidle PJ, Yang C, Ndiege K, Hamers RL, Rinke de Wit TF, Thomas T, Zeh C "Prevalence and dynamics of the K65R drug resistance mutation in HIV-1-infected infants exposed to maternal therapy with lamivudine, zidovudine and either nevirapine or nelfinavir in breast milk" J.Antimcrob Chem 2016, 71(6):1619-26

Inzaule SC, Hamers RL, Zeh CE, Rinke de Wit TF. "Stringent HIV viral load threshold for virological failure using dried blood spots: is the perfect the enemy of the good?" J Acquir Immune Defic Syndr 2016, 1; 71(1):e30-3

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Bosco A, Karamagi C, Kamya M, Rutazaana D, Kizito D, **Chekata S**, Nalusiba J, Gasasira A, Kalyango J, Mukoyo E, Nakamya P, Nankabirwa J, Katamba A, Byakika-Kibwika P, Meya D "Early evidence of reduction in all-cause and malaria attributed deaths at health facilities following scale-up of malaria interventions in Uganda 2004-2010" Journal of advanced biomedical studies, 2014: 1(1):001-007

CURRICULUM VITAE

Seth Chekata Inzaule (1985, Kenya) received his bachelors degree in biochemistry and molecular biology (first class honors) in 2007 from Jomo Kenyatta University of Agriculture and Technology, Nairobi, Kenya. His enthusiasm for infectious diseases and global health was ignited during his internship in the HIV research laboratory in the Kenya medical research collaborative program with the US Centers for Disease Control and Prevention. He then went on to work as a research assistant with particular focus on HIV drug resistance. He received his master's degree in clinical epidemiology and biostatistics in Makerere Uganda in 2012. He then went on to serve as a research officer with the HIV research laboratory and later as a tutorial fellow in Jaramogi Oginga Odinga University of Science and Technology, Kisumu, Kenya teaching epidemiology and biostatistics. Seth conducted his PhD research on determinants for achieving the third global "90" target for viral suppression in HIV infected persons in sub-Saharan Africa at the University of Amsterdam, Amsterdam UMC, the Amsterdam Institute for global health and development (AIGHD) and Barcelona university Institute for Global Health (ISGlobal) under the supervision of Promotores Prof. dr. Tobias Rinke de Wit and dr. Denise Naniche and Co-Promoter dr. Raph Hamers. He presented the key study findings at several local and international conferences between 2015 and 2018. He is currently working as a consultant with the WHO HIV/hepatitis program in the HIV treatment and care unit.