Drug-resistant HIV-1 in sub-Saharan Africa: clinical and public health studies

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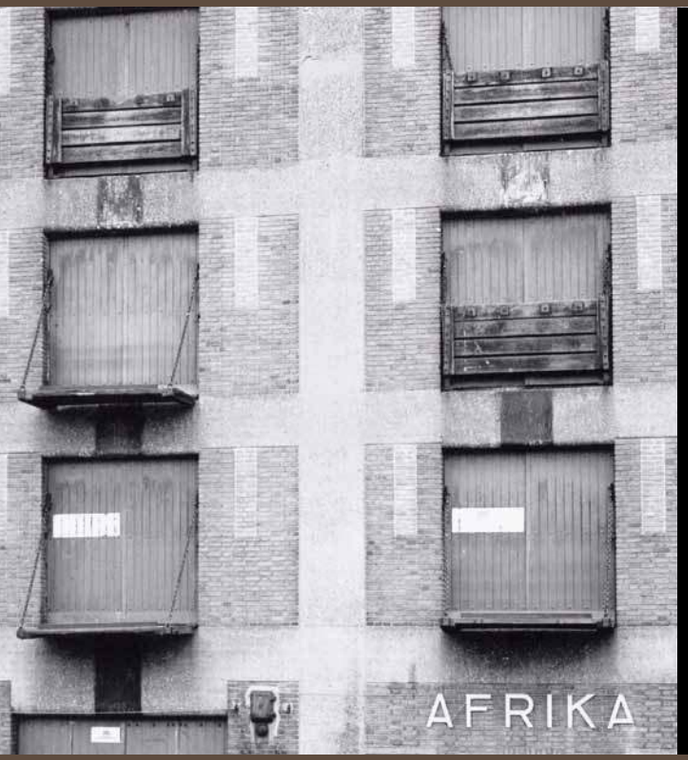

The past decade has witnessed an unparalleled expansion of access to antiretroviral treatment for people living with HIV/AIDS in sub-Saharan Africa. This historic public health achievement has saved the lives and improved the well-being of millions of people. Concern has been raised about rising drug-resistant HIV in resource-limited countries as a potential threat to the worldwide control of HIV/AIDS. To this end, the PharmAccess African Studies to Evaluate Resistance (PASER) network was established in Kenya, Nigeria, South Africa, Uganda, Zambia, and Zimbabwe in 2006. This thesis presents the results of landmark research on the epidemiology, diagnostic strategies, clinical management and public health implications related to emerging HIV drug resistance in sub-Saharan Africa.

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Drug-resistant HIV-1 in sub-Saharan Africa

Clinical and public health studies

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Abbreviations

3TC	lamivudine
ABC	abacavir
AIDS	Acquired Immunodeficiency Syndrome
AIGHD	Amsterdam Institute for Global Health and Development
ART	combination antiretroviral therapy
ART-A	Affordable Resistance Test for Africa
ARV	antiretroviral (drug)
ATV	atazanavir
AZT	zidovudine
bPI	ritonavir-boosted protease inhibitor
CHAI	Clinton HIV/AIDS Foundation
CI	confidence interval
CRF	circulating recombinant form; case report form
d4T	stavudine
DNA	deoxyribonucleic acid
DRM	drug resistance mutation
EFV	efavirenz
ETR	etravirine
EWI	early warning indicator
FBO	faith-based organization
GFATM	Global Fund to Fight AIDS, Tuberculosis and Malaria
FTC	emtricitabine
HIV	Human Immunodeficiency Virus
HIVDR	HIV drug resistance
HIVResNet	Global HIV Drug Resistance Network
IAS-USA	International Antiviral Society–USA
ICER	incremental cost effectiveness ratio
LAASER	Linking African and Asian Societies for an Enhanced Response to HIV/AIDS
LPV	lopinavir
MPR	medication possession ratio
NASBA	nucleic acid sequence-based amplification
NGO	non-governmental organization
NNRTI	non-nucleoside reverse transcriptase inhibitor
NRTI	nucleoside reverse transcriptase inhibitor

NVP	nevirapine
OR	odds ratio
PASER	PharmAccess African Studies to Evaluate Resistance
PASER-M	PASER-Monitoring
PASER-S	PASER-Surveillance
PCR	polymerase chain reaction
PDR	pretreatment drug resistance
PEPFAR	US President's Emergency Plan for AIDS Relief
PFP	private for profit
PI	protease inhibitor
PMTCT	prevention of mother-to-child transmission of HIV
PR	protease
pVL	plasma HIV viral load
RLP	rilpivirine
RNA	ribonucleic acid
RT	reverse transcriptase
RT-PCR	reverse transcriptase polymerase chain reaction
SDRM	surveillance drug resistance mutation
sd-NVP	single-dose nevirapine as PMTCT
TAM	thymidine analogue mutation
TAQAS	TREAT Asia Quality Assessment Scheme
TASER	TREAT Asia Studies to Evaluate Resistance
TDF	tenofovir
TDR	transmitted drug resistance
VAS	visual analogue score
VL	HIV viral load
WHO	World Health Organization


AFRIKA

Background


AFRIKA

Chapter 1

General introduction

HIV GLOBAL EPIDEMIC

The first cases of a novel immunodeficiency syndrome were reported in San Francisco, US, in 1981 [1]. In 1983, the causative agent was discovered to be a novel retrovirus, which was named Human Immunodeficiency Virus (HIV) [2]. The syndrome was named Acquired Immunodeficiency Syndrome (AIDS). Since the 1980s, the HIV/AIDS epidemic has had a detrimental impact worldwide. To date, HIV/AIDS has claimed the lives of more than 25 million people, and more than 30 million people are estimated to be living with HIV/AIDS worldwide [3]. Sub-Saharan Africa is the region most heavily affected by HIV/AIDS, accounting for 68% of all people living with HIV/AIDS, 70% of all new HIV infections in 2010, and 72% of all AIDS-related deaths [3]. The epidemic is most severe in southern Africa. Recently, the largest epidemics in the region —in Ethiopia, Nigeria, South Africa, Zambia, and Zimbabwe— have either stabilized or are showing signs of decline [3]. The number of annual AIDS-related deaths is steadily decreasing, reflecting the introduction of combination antiretroviral therapy (ART) as well as a decreasing incidence [3].

HIV is an enveloped retrovirus that belongs to the genus of lentiviridae, which is subdivided in two types: HIV-1, most common globally, and HIV-2, a less pathogenic variant concentrated in west Africa. HIV-1 has been divided into four distinct genetic groups: M, N, O and P [4-6]. Group M (major) is responsible for over 90% of HIV-1 infections globally. Natural genetic variation has led to the sub-classification of HIV-1 group M into nine subtypes (A-D, F-H, J, and K) and numerous circulating recombinant forms (CRFs) [7]. Subtype B is most prevalent in Europe, North America and Australia [8]. In sub-Saharan Africa, HIV-1 subtype C is responsible for 56% of infections, mainly in southern and east Africa, whereas smaller proportions of infections are caused by subtypes A, D, G, CRF_AG and other CRFs [8].

ANTIRETROVIRAL TREATMENT

HIV-1 can be transmitted sexually, through parenteral exposure to blood, or from mother to child during pregnancy, birth or breast-feeding. HIV-1 primarily targets CD4+ T-lymphocytes and macrophages. The acute infection is characterized by a burst of viral replication and immune activation [9, 10] and followed by a symptom-free interval of on average eight to ten years. During chronic infection, the number of CD4+ T-lymphycytes gradually declines. Without ART, cell-mediated immunity will eventually be lost and the immunodeficient HIV-infected individual becomes susceptible to opportunistic infections and neoplasms. The final stage, AIDS, will lead to death, if untreated.

ART reduces HIV-related morbidity and mortality by lowering of the viral load to minimum levels, thereby allowing the immune system to recover and preventing opportunistic infections [11, 12]. Notably, with currently available treatment modalities, HIV-1 cannot be eliminated from infected persons. Since ART became available in 1996, HIV no longer inevitably leads to AIDS and death. As a result of social mobilization, high-level political commitment and substantial international funding during the past decade, access to ART in resource-limited countries has been rapidly scaled-up. By the end of 2010, more than five million people in sub-Saharan Africa were receiving ART, reaching nearly 50% of those in immediate need [3]. HIV-related morbidity and mortality have significantly decreased for individuals receiving ART in the region [11, 13]. Despite these impressive gains, access is still not universal, and the United Nations General Assembly has recently committed to a target of treating 15 million people by 2015 worldwide [14].

To allow the scale-up of ART in resource-limited countries, a public health approach, developed by the World Health Organization (WHO) has been critical [15]. This approach is based on simplified ART protocols, including standard first-line and second-line ART regimens, limited laboratory monitoring, and a decentralized service delivery. Standard first-line regimens consist of a single non-nucleoside reverse transcriptase inhibitor (NNRTI) and a dual nucleoside/nucleotide reverse transcriptase inhibitor (NRTI) backbone, often available as generic fixed-dose combinations [16, 17]. Recommended second-line regimens combine a ritonavir-boosted protease inhibitor (bPI) with two previously unused and/or recycled NRTIs [16, 17], although availability of second-line regimens is still restricted in many settings. Because of resource constraints, plasma viral load testing is not generally available to monitor therapy effectiveness and detect therapy failure. Instead, WHO-defined HIV clinical staging and –if available– CD4 cell counts are commonly used to guide decision-making about regimen switching [16, 17].

HIV DRUG RESISTANCE

HIV-1 infection is characterized by high genetic diversity of the virus. This is the result of high levels of viral replication [18, 19] coupled with a high mutation frequency in the HIV genome, which is generated by error-prone reverse transcription during the HIV replication cycle [20, 21]. Consequently, a large pool of genetically related but distinct genetic virus variants, called quasispecies, are present within the infected individual [22]. Distinct quasispecies may have either deleterious mutations, mutations that reduce their fitness, or mutations that provide a fitness advantage in a particular environment, such as in the presence of antiretroviral drugs. Mutations (i.e. point mutations, insertions or deletions) may result in changes in the amino acid coding of the HIV proteins,

potentially altering the structure and/or function of these proteins and affecting the fitness of the viral strain. Mutations can cause drug resistance by structural alteration of the target molecule that prevents or reduces inhibitor binding (in the case of PIs, NRTIs, NNRTIs, and entry inhibitors), or by directly affecting the mechanism of action of the reverse-transcriptase enzyme (in the case of NRTIs).

ART applies a combination of antiretroviral agents from different drug classes to minimize the risk of drug resistance development. An effective ART regimen will suppress the replication of most quasispecies. However, quasispecies containing one or more resistance-associated mutations may continue to replicate at a very low rate. In the presence of the selective pressure of ART, viruses with reduced susceptibility to one or more of the drugs in the regimen will out-compete the wild-type (i.e. drug-susceptible) virus, and eventually become predominant in the quasispecies population. If the selective pressure of ART is removed, the virus variants harbouring resistance may be replaced by more efficiently replicating wild-type virus [23]. The drug-resistant variants persist as minority quasispecies, archived in the proviral DNA, and may re-emerge if selective pressure is re-applied by restarting the antiretroviral drug that selected for them. Overall, mutation and selection is a dynamic process determined by the potency of the ART regimen, drug concentrations, cross-resistance, and the effects of resistance on viral fitness.

Drug-resistant HIV-1 variants that are selected by ART during residual viral replication, called acquired drug resistance, constitute a reservoir for onward transmission to newly infected individuals, called primary or transmitted drug resistance (TDR) [24, 25]. Individuals who are newly infected with a drug-resistant variant can further contribute to the spread of drug-resistant HIV-1 [26, 27]. Although TDR variants may persist in untreated individuals [28], they may revert to wild-type virus over time or diminish to levels below detection by population-based genotyping [29]. Some of the challenges in studying the epidemiology of TDR include differences between studies in the definition and interpretation of resistance test results, duration of HIV-1 infection, time period, geographic region, subpopulation, and the possibility of undisclosed previous exposure to antiretroviral drugs.

SCALE-UP OF ANTIRETROVIRAL TREATMENT IN RESOURCE-LIMITED SETTINGS

In resource-limited countries, concern has been raised about the potential emergence and spread of HIV-1 drug resistance and its public health implications after the scale-up of antiretroviral drugs. In Europe and North America, the wider use of ART has been as-

sociated with an increase in levels of TDR [30-32], peaking in some settings at over 20% before levelling off at 9-15% in the era of (highly active) ART [24, 25, 33, 34]. Notably, its evolution has occurred in the context of (non-potent) sequential mono and dual therapies of NRTIs before 1996 [30-32]. By contrast, in resource-limited countries, the history and conditions of HIV treatment have been very different. The rapid scale-up of ART since 2003-2004 has been rightfully given priority to save the lives of millions of HIV-1 infected Africans, using potent, triple combination therapy from the onset. Relatively little attention has been paid to the development and spread of drug-resistant HIV-1 as a potential consequence of the widespread distribution of ART.

Factors contributing to HIV-1 drug resistance in resource-limited countries can be broadly grouped into four categories: regimen- and drug-specific, virus-related, patient-specific, and programmatic. A recognized limitation of NNRTI-based regimens is their relatively lower genetic barrier to resistance when compared to bPI regimens. Suboptimal regimens, such as the peripartum use of single-dose nevirapine to prevent mother-to-child HIV transmission (PMTCT), drug-drug interactions, inappropriate prescribing practices, use of non-quality assured drugs can further increase the risk of acquiring drug resistance [35]. For example, concomitant use of rifampicin in tuberculosis co-infected patients has been shown to reduce levels of nevirapine [36]. Poor adherence to ART is a predictor of virological failure [37-41], drug resistance, disease progression [42-44] and death [45]. Programme-level factors, such as limited human resources, inadequate infrastructure and weak supply management systems, can also negatively affect treatment adherence, retention in care and ultimately facilitate the emergence of population-level HIV drug resistance. Fragile drug procurement and supply management systems can result in drug stock-outs [46]. The absence of routine viral load monitoring, which is a more sensitive indicator of treatment failure than clinical-immunological parameters, may lead some patients to experience prolonged periods of virological failure prior to change of regimen [47, 48]. Moreover, although current evidence is limited, it has been suggested that the propensity to develop drug resistance and the spectrum of mutations that are acquired during ART, may differ across the various HIV-1 subtypes and CRFs [49].

The threat of increased TDR after the ART scale-up in sub-Saharan Africa has the potential to compromise the effectiveness of first-line ART regimens. Therefore, a new challenge that may confront national HIV treatment programs is how to manage emerging drug-resistant HIV-1. However, few data exist to adequately inform policy.

RESEARCH SETTING: THE PASER NETWORK

In 2006, a collaborative bi-regional program was established in sub-Saharan Africa and Asia, (*Linking African and Asian Societies for an Enhanced Response to HIV/AIDS*, denoted LAASER) with the primary aim of developing the regional capacities for the population-based assessment of acquired and transmitted HIV-1 drug resistance, thereby advancing the epidemiological, clinical and laboratory knowledge of the management of drug resistance in the regions. LAASER received financial support from The Netherlands Ministry of Foreign Affairs in partnership with Stichting AidsFonds (2006-2011).

As part of LAASER, the *PharmAccess African Studies to Evaluate Resistance* (PASER) network was established as a collaborative partnership of clinical sites, laboratories and research groups in Kenya, Nigeria, South Africa, Uganda, Zambia, and Zimbabwe. Table 1 summarizes some relevant country characteristics. PASER has implemented two laboratory-based study protocols: prospective cohorts to assess pre-therapy and acquired resistance in patients receiving first- or second-line ART (Monitoring, PASER-M), and cross-sectional surveys to assess TDR in recently HIV-1 infected populations (Surveillance, PASER-S). PASER contributes to fulfilling the goals of the Global HIV Drug Resistance Network (HIVResNet), developed by the WHO (http://www.who.int/hiv/topics/drugresistance/hivresnet). The implementation of HIV-1 drug resistance surveys in resource-limited countries is challenged by the high cost and complexity of genotypic resistance testing. To address this issue, PASER has initiated a public–private consortium, called *Affordable Resistance Test for Africa* (ART-A), which aims to develop a more affordable test algorithm for HIV-1 drug resistance. The studies included in this thesis were conducted as part of the PASER and ART-A programs.

Table 1. HIV/AIDS characteristics of PASER countries (source: WHO/UNAIDS)

Country	Number of people infected with HIV (millions) (2009)	National adult HIV prevalence (2009)	Year of introduction of national HIV treatment program	Number of people receiving ART (2010)	National ART coverage (2010)
Kenya	1.5	6.3%	2003	432,621	61%
Nigeria	3.3	3.6%	2002	359,181	26%
South Africa	5.6	17.8%	2004	1,389,865	55%
Uganda	1.2	6.5%	2000	248,222	47%
Zambia	1.0	13.5%	2003	344,407	72%
Zimbabwe	1.2	14.3%	2004	326,241	59%

RESEARCH OBJECTIVES

The aim of this thesis was to study the extent of HIV-1 drug resistance and its potential public health implications after the scale-up of ART in sub-Saharan Africa.

The research objectives of the thesis were:

- To define the epidemiology of TDR in HIV-1 infected populations after the scale-up of ART.
- To assess the effects of pre-therapy HIV-1 drug resistance on the response to first-line or second-line ART in routine ART programs.
- To assess patterns of HIV-1 drug resistance mutations and their clinical impact in patients experiencing failure of standard first-line or second-line ART in routine ART programs.
- To explore the implications of emerging HIV-1 drug resistance for public health policy in resource-limited countries.

OUTLINE OF THESIS

Background

As an introduction to the thesis, the first three chapters include a review of data on HIV-1 drug resistance in sub-Saharan Africa that were available before the start of the PhD research (**Chapter 2**), including an illustrative patient case study (**Chapter 3**), and a profile of the PASER-M cohort (**Chapter 4**).

The core of the thesis comprises three parts that include studies on epidemiological (**Part I**), clinical (**Part II**) and public health (**Part III**) aspects of drug-resistant HIV-1 in sub-Saharan Africa.

Transmitted HIV-1 drug resistance (Part I)

The first part focuses on the epidemiology of TDR in various countries and populations after the scale-up of ART, based on data from the PASER-M and PASER-S studies. The first study compares the prevalence and patterns of pre-therapy resistance between antiretroviral-naïve and antiretroviral-exposed individuals in Lusaka, Zambia, who are about to start standard first-line ART (**Chapter 5**). Subsequently, we assess TDR prevalence in antiretroviral-naive adults from 11 regions in Kenya, Nigeria, South Africa, Uganda, Zambia, and Zimbabwe, and examine if wider use of ART in sub-Saharan Africa is associated with rising prevalence of TDR (**Chapter 6**). Finally, we assess TDR in a recently HIV-1 infected population in Kampala, Uganda (**Chapter 7**).

Antiretroviral treatment and acquired resistance (Part II)

The second part includes clinical studies on therapy response and drug resistance patterns in patients receiving first-line ART (**Chapters 8 and 9**), switching to second-line ART (**Chapter 10**), or receiving second-line ART (**Chapter 11**), who are enrolled in 13 regular ART programs in six African countries. All studies are based on data from the PASER-M cohort. The first study prospectively assesses the effect of pre-therapy resistance on the immunological, virological and resistance outcomes of first-line ART (**Chapter 8**). Subsequently, the patterns of drug resistance mutations in patients experiencing virological failure after 12 months of first-line ART are described, including the implications for second-line therapy strategies (**Chapter 9**). In **Chapter 10**, we investigate patients at time of switch to a second-line regimen who experienced prolonged first-line failure, in the absence of plasma viral load monitoring. Particularly, we assess the diagnostic accuracy of clinico-immunological failure criteria –i.e. the proportion of patients who are misdiagnosed with virological failure and switched unnecessarily–, and the patterns of HIV-1 drug resistance mutations that are present at time of switch after prolonged failure. Finally, we prospectively assess the response to empiric second-line ART, including the effect of first-line resistance, and the patterns of drug resistance mutations in patients failing second-line ART (**Chapter 11**).

Public health policy (Part III)

The third part expands on the implications of emerging drug-resistant HIV-1 for clinical practice and public health policy in resource-limited countries. First, we discuss the operational experiences, achievements and challenges in establishing the PASER network (**Chapter 12**), and report the results of a site-level assessment of WHO-recommended early-warning indicators of HIV-1 drug resistance in all clinical sites collaborating in the PASER network (**Chapter 13**). In **Chapter 14**, we report a model-based analysis on the costs, life-expectancy and cost-effectiveness associated with laboratory-based diagnostic monitoring of patients receiving ART in sub-Saharan Africa using either CD4 cell counts or plasma viral loads only, as compared to clinical monitoring. In **Chapter 15**, a systematic review was undertaken of the usefulness and limitations of dried fluid spots as a practical, affordable specimen matrix to measure HIV-1 viral load and genotypic resistance in resource-limited countries. Finally, we expound recommendations and priorities for public health policy in view of rising drug-resistant HIV-1 in sub-Saharan Africa (**Chapter 16**).

Discussion

The final chapter (**Chapter 17**) is a summary and general discussion of the main research findings of this thesis, followed by some concluding remarks.

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AFRIKA

Chapter 2

The 2008 status of HIV-1 resistance to antiretroviral drugs in sub-Saharan Africa

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ABSTRACT

Access to combination antiretroviral therapy (ART) for persons infected with HIV in sub-Saharan Africa has greatly improved over the past few years. However, data on long-term clinical outcomes of Africans receiving ART, patterns of HIV resistance to antiretroviral drugs and implications of HIV type-1 (HIV-1) subtype diversity in Africa for resistance, are limited. In resource-limited settings, concerns have been raised that deficiencies in health systems could create the conditions for accelerated development of resistance. Coordinated surveillance systems are being established to assess the emergence of resistance and the factors associated with resistance development, and to create the possibility for adjusting treatment guidelines as necessary. The purpose of this report is to review the literature on HIV-1 resistance to antiretroviral drugs in sub-Saharan Africa, in relation to the drug regimens used in Africa, HIV-1 subtype diversity and overall prevalence of resistance. The report focuses on resistance associated with treatment, prevention of mother-to-child transmission and transmitted resistance. It also outlines priorities for public health action and research.

INTRODUCTION

The use of combination antiretroviral therapy (ART) in individuals infected with HIV type-1 (HIV-1) has effectively reduced morbidity and mortality in the industrialized world [1]. The implementation of ART in developing countries with a high HIV prevalence, including the hardest-hit area of sub-Saharan Africa (herein referred to as Africa), is a global public health priority [2]. The number of HIV-infected persons in Africa who have access to ART is estimated to have increased 10-fold over the past 3 years. By December 2006, it was estimated that more than 1.3 million Africans had received ART, reaching 28% of those in need, yet leaving over 70% without access [3].

Deficiencies in health systems and resources, such as unreliable supply systems, shortage of staff and lack of virological monitoring, could create the conditions for accelerated development of HIV-1 resistance to antiretroviral drugs. Patients receiving antiretroviral drugs could acquire resistance, and subsequently transmit resistant viruses to newly infected persons. High rates of resistance could eventually compromise the effectiveness of antiretrovirals in the general population [4–6]. Moreover, the high diversity of HIV-1 non-B subtypes prevalent in Africa [7] might have implications for the patterns of resistance development.

The purpose of this report is to review the literature that describes HIV-1 resistance to antiretroviral drugs in Africa, in relation to the drug regimens used in Africa, HIV-1 subtype diversity and overall prevalence of resistance. The report focuses on resistance associated with treatment, prevention of mother-to-child transmission (pMTCT) and transmitted resistance, and outlines priorities for public health action and research.

METHODS

To identify eligible studies, a systematic search of the English language literature published before 2008 was conducted. The search included the Medline database, relevant treatment guidelines, the World Health Organization (WHO) website and abstracts presented at international conferences. The search strategy combined the terms 'antiretroviral therapy', 'public health', 'drug resistance', 'surveillance', 'HIV-1 subtype diversity' and 'sub-Saharan Africa'.

RESULTS

Principles of resistance and WHO treatment guidelines

Principles of resistance

The viral replication process of HIV-1 is exceedingly error-prone, leading to a high mutation frequency [8, 9]. In combination with a rapid viral turnover [10, 11], this results in a pool of genetically related but distinct viruses, called quasi-species, within each infected individual. The most frequently used antiretroviral drugs target the replication enzymes reverse transcriptase (RT) and protease (PR), which are encoded by the HIV-1 polymerase (*pol*) gene. Virus variants that have mutations at specific positions of nucleic acid in *pol* could be selected by drug selective pressure, leading to reduced susceptibility, or resistance, to that particular drug. Selection of resistant viruses occurs in the context of incomplete suppression of viral replication when optimal drug levels are not maintained, either through poor adherence, treatment interruptions or the use of suboptimal drug combinations (acquired resistance). For instance, single-dose nevirapine (SD-NVP), which is commonly used for pMTCT in HIV-infected pregnant women in Africa, is a non-suppressive regimen. A second method of acquiring resistance is via transmission of a resistant strain to a newly infected person (primary resistance). Virus variants harbouring resistance might replicate less efficiently than wild-type virus strains. In the absence of drug selective pressure, resistant viruses might be rapidly outgrown by wild-type virus strains which are fitter. As such, the mutant virus becomes undetectable in the plasma virus populations, but will still be archived in the proviral DNA population of HIV-1-infected cells, re-emerging only if drugs to which they are resistant are restarted [12]. Each antiretroviral drug or drug combination has its own resistance profile, which could be specific to the drug or could express cross-resistance to other drugs within the same class [13]. Drugs with a high genetic barrier, such as zidovudine (ZDV) and most protease inhibitors (PIs), require the accumulation of multiple mutations to overcome antiviral drug activity. On the other hand, drugs with a low genetic barrier, including lamivudine (3TC) and non-nucleoside reverse transcriptase inhibitors (NNRTIs), only require a single point mutation to confer high-level resistance.

WHO treatment guidelines

In view of the public health benefits of accelerating access to ART in resource-limited countries, the WHO has developed a public health approach to treatment based on standardized, simplified guidelines and a decentralized service delivery [14, 15]. In the absence of specialist physicians and extensive virological patient monitoring, which is the standard care model in industrialized countries, the public health model enables healthcare workers with minimum training to deliver care to large numbers of patients.

Clinical decision-making is guided by clinical observation, WHO clinical staging and, if available, haematology, biochemistry and CD4+ T-cell counts.

The standard ART regimens used in Africa are based on relatively inexpensive drugs, which are produced generically in large quantities and are often available in a fixed-dose combination. WHO guidelines include a standard first-line regimen consisting of either two nucleos(t)ide reverse transcriptase inhibitors (NRTIs) plus an NNRTI or a triple NRTI regimen, and a second-line regimen consisting of a boosted PI with at least one NRTI [14]. The most frequently used first-line regimen consists of the dual NRTI backbone (3TC and either ZDV or stavudine [d4T]) plus an NNRTI (either NVP or efavirenz [EFV]). ZDV and d4T are thymidine analogue drugs and both select for a common set of mutations called thymidine analogue mutations (TAMs). Accumulated TAMs induce cross-resistance to other NRTIs. Both 3TC and NNRTIs have a low genetic barrier, presenting a potential vulnerability of the current standard first-line therapy. Because of high costs, the availability of PIs in Africa has been limited, reserving them for second-line therapy only. Given that fewer regimens are available in resource-limited countries, it is of particular importance to minimize resistance.

HIV-1 subtype diversity and resistance

HIV-1 subtypes

HIV-1 has been divided into three distinct genetic groups: M, N and O [16]. Whereas groups N and O represent a small minority of HIV-1 infections in central Africa [17, 18], group M is responsible for over 90% of HIV-1 infections globally, comprising nine subtypes (A–D, F–H, J and K) [19] and a number of circulating recombinant forms (CRFs) [20, 21]. Subtype B is still the predominant subtype in Europe, North America and Australia, but is hardly found on the African continent, where all other (non-B) subtypes are represented with a distinct geographic distribution [7]. In Africa, subtype C is responsible for 56% of infections, mainly in the south and east, whereas smaller proportions of infections are caused by subtypes A (14%), G (10%), CRF02_AG (7%) and other recombinants (9%) [7].

Antiretroviral drugs that are currently available were developed on the basis of their activity to primarily inhibit the replication of subtype B viruses. As a result, scientific data on patterns of resistance and clinical outcome of ART is largely limited to this subtype. Preliminary data suggests that short-term immunological and virological outcomes on ART are similar for Africans compared with their Western counterparts [22, 23]. However, these results have been obtained with a limited set of first-line regimens and long-term outcome data is not yet available.

Nucleotide differences between subtypes could have an effect on the spectrum of amino acid substitutions resulting from point mutations, which in turn might influence the biochemical and biophysical microenvironment in the PR and RT *pol* gene regions [24–26]. As a result, intersubtype differences in the genes targeted by antiretroviral drugs could influence their primary drug susceptibility, the propensity to develop resistance and the spectrum of mutations that emerge during drug selective pressure, either as a consequence of the nucleotide composition at baseline or by the emergence of specific mutations during therapy.

Natural polymorphisms

Certain naturally occurring genetic variations, called polymorphisms, are frequently found in untreated populations infected with a non-B subtype of HIV-1. Analyses of drug-naive virus isolates of various non-B subtypes have shown that 53% and 48% of PR and RT positions, respectively, are naturally polymorphic, as compared to subtype B [27, 28]. In subtype B, some polymorphisms at specific amino acid residues (including PR positions 10, 20, 36, 63, 71, 77 and 93 and RT positions 69, 75, 98, 106, 118, 179 and 214) are known to be associated with resistance [27, 28]. The extent to which the abundance of polymorphisms in the non-B subtypes alters PR and RT function, drug susceptibility or clinical response to therapy is still unclear. For instance, the naturally occurring Y181C and Y181I genotypes in HIV-1 group O and HIV-2 render these viruses resistant to all NNRTIs [29, 30]. Some polymorphisms, such as the frequently occurring M36I in PR, could restore or support the replication capacity of resistant virus thereby facilitating the emergence of resistance under drug pressure [31]. Other data on the possible clinical consequences of inter-subtype differences in polymorphisms are inconclusive [32–37].

Mutational pathways

The most common resistance mutations reported in studies conducted in Africa are M184V and K103N, and are a consequence of the widespread use of 3TC and NNRTIs, respectively, as part of the standard first-line therapy. Both mutations also occur frequently in subtype B viruses. Indeed, there is currently no evidence that non-B viruses develop resistance by 'new' mutations, that is, at positions that have not been associated with resistance in subtype B viruses [26]. Although limited, the available data provides reassurance that, for the most part, the various subtypes share common mutational pathways of resistance. Moreover, a recent analysis concluded that the overall genetic barrier to resistance was similar for the various HIV-1 subtypes [38]. However, some subtype-related mutational pathways have been reported that might have implications for the African context. For instance, tenofovir (TDF) might select the K65R mutation more rapidly in subtype C compared with subtype B [39, 40]. In light of increasing and recommended use of TDF as part of first-line therapy in Africa, this finding could have

implications for therapy effectiveness. Also, several studies have demonstrated inter-subtype differences in frequency and long-term persistence of resistance mutations in women and infants after the use of SD-NVP for pMTCT [41–44]. Finally, *in vitro* EFV rapidly selects the V106M mutation in subtype C, as opposed to the Y181C mutation in subtype B [45]. This is explained by an inter-subtype difference in the genetic barrier to resistance: the wild-type V106A needs two nucleotide changes in subtype B, as opposed to only one in subtype C. Further investigations are warranted to identify additional inter-subtype differences in mutational pathways, to ascertain whether these are caused by the genetic differences between subtypes or are a result of other variations, such as differences in patient monitoring and therapy-switching policies, and to evaluate their effect on clinical outcome.

Genotypic algorithm interpretation

For the clinical interpretation of genotypic resistance data algorithms, such as those from Stanford, REGA and ANRS, are used which apply certain rules to determine the presence of mutations, and subsequently predict their effect on drug activity. However, algorithms used at present are mainly based on subtype B data. As a result, in non-B subtypes their reliability might be limited, as they do not take into account any possible inter-subtype differences in drug susceptibility and resistance evolution outcomes of known and new mutations [46, 47].

Treatment-associated resistance

Available data

Twelve studies reported rates of resistance among patients receiving ART in Africa. The studies were conducted in Uganda, Senegal, Zimbabwe, Rwanda, Cameroon, Botswana, Côte d'Ivoire and Tanzania [40, 48–58] (figure 1, table 1). Observational data from patients on a first-line ART regimen show large variations in the rate of resistance, reported at 3.7%–49% after 24–163 weeks on ART [40, 48–58]. Earlier studies showed that the use of non-suppressive regimens (mono or bi-therapy) with inappropriate therapeutic monitoring rapidly led to high levels of resistance [56, 59, 60]. However, comparison of study results is difficult because of dissimilarities in drug regimens used, previous use of antiretroviral drugs, duration of follow-up and HIV-1 subtypes. Overall, the reported resistance rates do not appear to exceed rates reported in industrialized countries, where the prevalence of resistance mutations has been estimated at 9% in patients after 2 years on ART, rising to 27% by 6 years [61]. Resistance outcome data on second-line regimens in Africa is virtually non-existent [40, 58]. A cohort study in Côte d'Ivoire has been the first to report data on clinical and immunological outcomes in African patients who are resistant. In patients who had a major resistance mutation by a median of 37

Figure 1. Map of sub-Saharan Africa showing the geographical location of studies that were included in the literature review.
Each symbol represents one study. Studies are subdivided by acquired resistance in patients receiving ART (stars), transmitted resistance (circles) and nevirapine resistance after single-dose nevirapine (squares).

months on ART, subsequent 20-month clinical and immunological outcomes were compromised when compared with patients who had no resistance [55]. Even less data is available on the prevalence of resistance in African children on ART and their clinical outcome [62–64]. Due to the success of pMTCT in industrialized countries, the bulk of this data will have to be generated in developing countries.

Contributing factors: inadequate health systems

After years of inadequate administration, insufficient funding and brain-draining, health systems in many African countries feature poorly functioning medical facilities and unreliable supply systems. Breakdowns in health systems create the conditions for accelerated resistance. Factors that most directly affect resistance arise from weak regulation, poor supply chain management (for example, for drugs and laboratory reagents), inadequate equipment maintenance arrangements, a lack of knowledge and training among providers and inadequate monitoring and control systems in hospitals and other care facilities [65, 66]. Moreover, Africa is facing a human resource crisis with serious

shortages of nurses and doctors, a problem that has been aggravated by the high rate of HIV infection among healthcare providers [67]. Ultimately, these weaknesses affect adherence to treatment regimens and quality of care, which are key factors in the prevention and containment of drug resistance.

Contributing factors: patient adherence to therapy

Meticulous adherence to therapy is considered the most important factor in the prevention of resistance [68–70]. Although the widespread introduction of fixed-dose combination drugs in developing countries has greatly simplified ART regimens, there are important sociocultural and environmental factors that pose barriers to the ability of patients to adhere to treatment. These also include the cost of regular transportation to the clinic and the challenge to afford the food needed to take with medicines. Several studies have reported poorer rates of patient retention and viral suppression, and higher mortality for fee-paying patients compared with patients who received their medication free of charge [22, 23, 71]. The risk of resistance development could be reduced by enhancing treatment adherence through uninterrupted drug supply and the provision of medical services, including medication and laboratory tests, at no or low cost. To eliminate barriers to adherence, adherence support and patient education by dedicated counselors should be emphasized [72]. There is a need for novel affordable methods to promote adherence specifically tailored to the sociocultural context of African adult and paediatric patients.

Contributing factors: prescribing patterns

Additional challenges to minimizing resistance include misdiagnosis, poor prescribing practices resulting from lack of training, sub-therapeutic dosage and the distribution of substandard drugs [66]. The availability of adequate second-line drug combinations is limited, leaving patients dependent on suboptimal drug combinations after failing the first-line therapy. The strong long-term side effects of some of the frequently used drugs, such as d4T, could negatively affect adherence, thus promoting resistance. Concomitant use of particular tuberculostatic agents (such as rifampin) could affect the blood levels of antiretroviral drugs such as PIs and EFV [73]. This is particularly relevant in view of the high rates of tuberculosis co-infection in Africa. Moreover, there is insufficient knowledge on potential interactions with other drugs.

Contributing factors: access to virological monitoring

There is insufficient laboratory capacity and financial resources in Africa to perform regular virological monitoring in patients on ART. Therapeutic monitoring based on clinical and immunological parameters alone might result in unnecessary switches to second-line ART in the absence of virological failure, but could also increase the risk that

patients will stay on a virologically failing regimen for longer periods [71,74]. This could result in accumulation of resistance mutations, which might compromise the efficacy of subsequent second-line therapy [75]. Once clinical failure arises, the ability to select an optimal treatment regimen will be further limited by the inability to test for resistance.

Transmitted resistance

Available data

The prevalence of transmitted resistance is highest in industrialized countries, estimated between 9% and 20% [5, 6, 76–78]. The WATCH study found that the rate of resistance (to any drug) among treatment-naive individuals was 5.5% in Africa [79]. Between 2002 and 2007, 19 studies reported rates of resistance among treatment-naive populations in Africa. Studies were conducted in South Africa, Zambia, Côte d'Ivoire, Malawi, Senegal, Botswana, Cameroon, Djibouti, Democratic Republic of Congo, Burundi, Mozambique, Burkina Faso and Tanzania [80–97] (figure 1, table 2). NNRTI resistance rates ranged from 0% to 5.6%, NRTI resistance ranged from 0% to 3.7% and primary PI mutations were rare. To date, most reports from Africa have described low rates of transmitted resistance to NRTIs and NNRTIs, which might reflect the restricted availability of antiretroviral drugs until recently. Most studies conducted in Africa have small samples and substantial dissimilarities in assay methodology, the time period in which data were collected, the population under study and HIV-1 subtypes, which limit generalizability and the possibilities for comparison.

Contributing factors

The most important risk factor for transmitted resistance seems to be widespread access to antiretroviral drugs in the area where infection occurred, particularly where drugs were used as part of non-suppressive regimens, such as industrialized countries before ART became available in 1996. By contrast, in Africa, where widespread treatment was only introduced when ART was available, it has been hypothesized that less resistant viruses are expected to circulate [76].

Mathematical modelling has shown that at currently planned levels of treatment coverage and unchanging sexual behaviour, ART rollout in Africa will not initially drive an epidemic of drug-resistant HIV [98]. However, if the assumptions made in the model (for example, those regarding ART coverage, level of transmission, rate of persistence of resistant viruses and replicative capacity of resistant viruses) are modified, it appears equally plausible that resistance transmission will have a substantial effect on disease epidemiology [99, 100]. Notably, recent studies have suggested that resistance acquired during HIV infection could persist over time. This might be due to the fact that the new

infection is caused by a relatively homogeneous virus population derived from the actively replicating virus population in the donor [101, 102]. This could not only impair the individual's response to treatment, but could also have an effect on the risk of becoming infected with resistant viruses that persist over time. Therefore, more sophisticated models are urgently needed to effectively inform policy.

pMTCT-associated resistance

In industrialized countries, the rate of mother-to-child transmission of HIV-1 has been reduced to <2% by the use of ART during pregnancy, elective caesarean delivery and avoidance of breastfeeding [103–105]. However, in the developing world, access to antenatal care is limited, leaving mother-to-child transmission the second major route of HIV infection and rendering the use of shorter and more practical regimens of NRTIs and/or NNRTIs for pMTCT widespread. Peripartum administration of SD-NVP to the mother at the onset of labour and to the infant at 48–72 h of life has been shown to be an easy and low-cost intervention, reducing HIV-1 transmission by 41%–47% [106, 107].

Data on resistance in women and infants following SD-NVP

SD-NVP, which has a low genetic barrier and a long half-life, does not provide maximum viral suppression, inducing the selection of resistance mutations in mothers and infants. Thirteen studies evaluated NVP resistance following SD-NVP. Studies were conducted in Côte d'Ivoire, South Africa, Uganda, Malawi and Zimbabwe. The most common resistance mutations were K103N and Y181C. Resistance rates ranged from 19% to 69% in women and from 40% to 87% in infants, with possible variations between subtypes [41, 42, 44, 108–117] (figure 1, table 3).

Data on resistance in women following other pMTCT regimens

Several studies have examined the emergence of resistance following other pMTCT regimens. A randomized trial comparing women receiving SD-NVP alone with women who received SD-NVP followed by either 3 or 7 days of ZDV and 3TC post-partum found that the prevalence of NVP resistance in these three groups was 57%, 13% and 9%, respectively [114]. Similarly, a non-controlled study found that the rates of NVP resistance in women were reduced when SDNVP was followed by the administration of ZDV plus 3TC for 3 days post-partum [118]. Accordingly, revised WHO pMTCT guidelines for resource-limited settings recommend the use of a combination of ZDV and 3TC post-partum, in addition to SD-NVP, in order to reduce the risk of NVP resistance [119]. A recent study from Zambia showed that a single dose of TDF and emtricitabine at delivery, in addition to SD-NVP and a short course ZDV, reduced NVP resistance in women by half at 6 weeks after delivery [120]. A recent meta-analysis reported NVP resistance rates at 4–8 weeks post-partum of 35.7% in women receiving SD-NVP with or without other ante

or intrapartum antiretrovirals, and 4.5% in women receiving SD-NVP plus post-partum antiretrovirals [121].

Data on resistance in infants following other pMTCT regimens

A number of studies evaluated resistance following other pMTCT regimens in infants. Mother-infant pairs who were treated with ZDV or SD-NVP showed NVP resistance in half of the pairs receiving SD-NVP and no ZDV mutations in those receiving ZDV at 6 weeks post-partum [122]. Infants who received SD-NVP plus 7 days of ZDV and 3TC showed no NVP resistance at 6 weeks compared with 78% of those who received SD-NVP only [114]. NVP resistance in infants could be reduced by adding a short-course of ZDV postpartum [123]. A recent meta-analysis reported NVP resistance rates at 4–8 weeks post-partum of 52.6% in infants receiving SD-NVP only and 16.5% in infants with additional post-partum antiretrovirals [121].

Clinical consequences of previous pMTCT

The clinical consequences of NVP exposure on effectiveness of NNRTI-based ART and/or pMTCT in later pregnancies are still unclear. Studies have reported that SD-NVP decreased the virological response of women to subsequent NVP-containing ART at 6 months [124, 125]. Others have suggested that effectiveness was not compromised at 18 months of follow-up [126] and that initial virological response was also not compromised if ART was started more than 6 months after delivery [125]. Furthermore, preliminary data suggest that there is no increase in NVP resistance when SD-NVP is taken for a second time in a subsequent pregnancy [127], and that effectiveness of SD-NVP for pMTCT used in successive pregnancies is probably not impaired [128,129]. Additional randomized trials are needed to definitively answer these questions. Meanwhile, because relatively few women (11% of those eligible [3]) are currently receiving SD-NVP and because most women will not immediately initiate ART following SD-NVP, WHO guidelines recommend that HIV-infected mothers and infants who require ART and have previously been exposed to SDNVP should still be considered eligible for NNRTI based regimens [119].

Priorities for public health action and research

As the number of individuals on ART across the African continent grows, the main challenge is to maintain the momentum in the rollout of treatment and prevention programmes achieved so far and to sustain those already in care. The next challenge will be to develop more effective and sustainable health systems, which include the appropriate infrastructure for logistics, administration, information management, laboratories and other facilities [130], and to take specific measures to prevent and contain resistance and to improve the quality of HIV care and treatment.

Preserving first-line regimens

Due to limited availability of virological monitoring, detection of resistance mutations and second-line therapy, prolonging the clinical efficacy of first-line therapy will be crucial [131]. Meticulous adherence to therapy must therefore be emphasized [68–70, 72]. Clinical trials evaluating which therapeutic monitoring strategies are essential to ensure long-term effectiveness of ART in resource-limited countries are ongoing. In addition, data are needed to determine the optimal time to switch from first-line to second-line therapy in the absence of resistance testing and salvage regimens.

Coordinated surveillance of resistance

Currently, in developing countries, the emergence of acquired and transmitted resistance is not routinely evaluated as part of treatment programmes. The coordinated assessment of the proportion of HIV-infected individuals who have developed resistance, patterns of resistance and the factors associated with resistance emergence and spread, will provide crucial information for adjusting treatment guidelines as necessary. To this end, the WHO launched a global public health strategy through the Global HIV Drug Resistance Surveillance Network (HIVResNet) and national governments [132]. Although the validity of the proposed study methodologies, which include early warning indicators, sentinel monitoring and threshold surveillance, needs to be confirmed, an important first step has been taken towards standardization and coordination of resistance surveillance efforts. The PharmAccess African Studies to Evaluate Resistance (PASER) programme is a major contributor to the global public health strategy in Africa. Together with its counterpart programme in Asia, TREAT Asia Studies to Evaluate Resistance (TASER), PASER aims to build capacity for coordinated resistance surveillance by establishing a network of HIV clinics, reference laboratories and research centres that collaborate in an observational resistance database [133]. Results are expected to support recommendations to policy makers for optimal ART practices.

Improved laboratory capacity

Over time, laboratory capacity in Africa should be improved to expand access to laboratory-dependent patient monitoring strategies, such as haematology, biochemistry, CD4+ T-cell counts and viral load testing, as feasible technologies become available [131]. Currently, the use of conventional resistance detection methods, mainly genotypic and phenotypic assays [134], are limited by prohibitively high costs, high capital outlay and significant technical skill required to conduct the assays. At present, WHO does not recommend resistance testing for individual patient management in resource-limited settings. The development of affordable and more practical alternatives for laboratory monitoring tools, including resistance assays, simple specimen carrier devices, in-house genotyping protocols and point mutation assays, should be pursued actively. As part

of the coordinated surveillance efforts, there is a need to build the laboratory capacity for quality-assured genotypic resistance testing. To this end, it seems most feasible to adopt a centralized approach with a limited number of regional reference laboratories in strategic African countries. Both HIVResNet and PASER are currently supporting the set-up of the appropriate infrastructure, including quality assurance schemes.

DISCUSSION

Breakdowns in health systems might create the conditions for accelerated emergence of antiretroviral resistance in resource-limited countries. The main contributing factors include interrupted drug supply, poor adherence to therapy, suboptimal prescribing patterns and limited access to virological monitoring. Studies conducted in Africa to date reported low rates of transmitted resistance, but predictions for the future are difficult to make. The use of non-suppressive drug regimens in HIV prevention strategies, such as in pMTCT, and the possible future use of microbicides and pre-exposure prophylaxis, warrants careful investigation of their consequences for resistance development.

This literature review was limited by the quality and quantity of the available studies. Small and selected samples in many studies meant data could not be easily extrapolated to the general population. Also, because of heterogeneity in study design, populations under study, HIV-1 subtypes and time of data collection, the possibilities of study comparison are limited.

In view of the numerous risk factors, the public health community should anticipate the realistic possibility of exacerbated emergence of resistance among African HIV-infected populations, as treatment and prevention programmes are scaled up. The containment of resistance in Africa is particularly important given the limited number of drug regimens that are available. Many important questions concerning patterns and prevalence of resistance, therapeutic monitoring strategies and implications of subtype diversity and pMTCT, remain to be definitively answered. The next main challenge is to vitalize the health systems and to take specific measures to minimize resistance. To this end, coordinated resistance surveillance systems are being established throughout the developing world.

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Table 1. Summary of studies on drug resistance in patients receiving ART in sub-Saharan Africa.

Year	Location	n	Study design	Study group	Median baseline CD4 count	Main HIV-1 subtypes	Main ART regimen	Median ART duration (weeks)	Comment	Ref
1998-2000	Uganda	94	CSA	Failing patients on ART for >90 days, subset of DAI cohort	73	A, D	2NRTIs vs. triple ART	36	Virologic failure (>400cps): 2NRTIs 20% vs ART 50% Overall HIVDR: 2NRTIs 29/37 (78%) vs. ART 22/45 (49%)	[56]
1998-2000	Senegal	58	PC	ARV naïve, advanced disease, subset of ISAARV cohort	109	CRF02_AG	d4T+ddI+IDV	78	Virologic failure (>500cps): 41% Overall HIVDR 2/58 (3%)	[50]
2001	Zimbabwe	25	CSA	Failing patients on ART for >2 months	95 *	C	Triple therapy, mostly PI-based; 1st and 2nd line	48	Virologic failure (>400cps): 21/25 (84%) Genotyping 21/25, HIVDR 17/21 (NRTI 82%, NNRTI 18%, PI 41%, ≥1 drug class 59%)	[58]
2002	Rwanda	60	CSA	Failing patients on ART for >3 months	na	A, C	91% triple therapy, mostly NNRTI-based	na	50% treatment interruption Virologic failure (>1000cps) 26/60 (43%) Genotyping 22/26, HIVDR 11/22 (NRTI 63%, NNRTI 55%, PI 27%)	[49]
2001-2003	Cameroon	109	PC	ARV naïve, ≥18yrs, CD4 <350/mm3 or AIDS, Karnofsky >50%	150	NA	3TC+NVP+ZDV or d4T	70	Virologic failure (>400cps): 18% at 24 months Overall HIVDR 4/109 (3.7%) Incidence: 3.2 per 100 person yrs	[48]
2003	Uganda	137	CSA	Failing patients on ART for >12 weeks, mostly ARV naive at baseline	163 *	A, D	Triple therapy, mostly NNRTI-based	38	Virologic failure (>400cps) 46/137 (34%) Genotyping 36/46, HIVDR 30/36 (mostly K103N)	[53]

Table 1 (continued)

Year	Location	n	Study design	Study group	Median baseline CD4 count	Main HIV-1 subtypes	Main ART regimen	Median ART duration (weeks)	Comment	Ref
2003	Uganda, Zimbabwe	377	CT	DART: ARV naïve, advanced disease	101	A, C, D	3TC+ZDV+TDF	24	Virologic failure (>1000cps) 53/377 (14%) Genotyping 20/53, HIVDR 18/20 (mostly M184V, K65R less common)	[54]
1998-2004	Senegal	176	CSA	Failing patients, mostly AIDS and ARV naïve	144	NA	2NRTIs+NNRTI or PI	131	Virologic failure (>500cps) 22.5% at 30 months Overall HIVDR 22/176 (13%) (NRTI 10%, NNRTI 9%, PI 8%)	[51]
2002-2004	Cameroon	128	CSA	Failing patients on ART for >3 months	NA	CRF02_AG	2NRTIs+NNRTI or PI	44	Genotyping 35/128, HIVDR 21/35 (NRTI 13% (mostly M184V), NNRTI 10% (mostly K103N), PI 2%)	[52]
2002-2005	Botswana	155	CSA	Failing patients	96	C	ddI+d4T+NFV, NFV-based 2nd line regimen	57	Virologic failure 16/155 (10%) Suggest subtype-C specific NFV resistance pathways (D30N 54%, L90M 31%)	[40]
2004-2006	Ivory Coast	106	CSA	Failing patients in ACONDA/ISPED cohort	122	NA	2NRTIs+NNRTI or PI	163	Virologic failure (>300cps/ml) 44/106 (42%) Overall HIVDR 23/106 (22%) (≥1 drug class 30%)	[55]
2005	Tanzania	150	CSA	Failing patients on ART for median ≥6 months	114	A, C, D	3TC +d4T+NVP	52	Virologic failure (>1000 cps/ml) 35/150 (23%) Genotyping 27/35, HIVDR 15/27 (NRTI 9%, NNRTI 10%)	[57]

Table sorted by year of study. *CD4+ T-cell count at time of cross-sectional analysis (CSA), not baseline. ARV, antiretroviral; CT, clinical trial; ddI, didanosine; d4T, stavudine; ART, combination antiretroviral therapy; HIVDR, HIV drug resistance; IDV, indinavir; NA, not available; NFV, nelfinavir; NNRTI, non-nucleoside reverse transcriptase inhibitor; NRTI, nucleoside reverse transcriptase inhibitor; NVP, nevirapine; PC, prospective cohort; PI, protease inhibitor; PR, protease; RT, reverse transcriptase; TDF, tenofovir; VF, virological failure; ZDV, zidovudine; 3TC, lamivudine.

Table 2. Summary of studies on rates of transmitted drug resistance in sub-Saharan Africa [a].

Year	Location	n	Study group	Median CD4 count	Main HIV-1 subtypes	Reported resistance rate (%)*					Mutations	Ref
						Any	NRTI	NNRTI	PI	MDR		
NA	Abidjan, Ivory Coast	20	ARV naïve (DAI cohort)	84	CRF02_AG	0	0	0	0	0		[80]
2000	Soweto, South Africa	37	Antenatal clinic attendees, ARV naive	479	C	0	0	0	0	0		[89]
2000	Lusaka, Zambia	28	Antenatal clinic attendees in first pregnancy	NA	C	0	0	0	0	0		[84]
1997-2000	Abidjan, Ivory Coast	99	Regulars, volunteers and blood donors, estimated time since seroconversion 9.4 months	NA	CRF02_AG	0	0	0	0	0		[91]
1996-2001	Lilongwe and Blantyre, Malawi	21	ARV naive, STD clinic and hospital attendees	NA	C	0	0	0	0	0		[88]
1998-2001	Dakar, Senegal	41	ARV naive subset (SIAARV cohort)	112	CRF02_AG	0	0	0	0	0		[93]
2001	KwaZuluNatal, South Africa	56	ARV naive, heterogenous	366	C	5.4	0	5.4	0	0	RT: G190A, K103N, A98G	[83]
2001	11 health districts, Botswana	71	Sentinel survey among antenatal and STD clinic attendees	NA	C	0	0	0	0	0		[82]
2001-2002	Abidjan, Ivory Coast	107	Blood donors (PRIMO-CI) and ARV naïve women (DITRAME Plus)	395	CRF02_AG	5.6	0.9	3.7	0.9	0	RT: K101E, K103N, P236L. K219Q PR: N88D	[92]
2000-2002	6 rural villages, Cameroon	128	Random subset of HIV diagnostic samples	NA	CRF02_AG	0	0	0	0	0		[86]
2002	Djibouti	47	Subset of general population survey	NA	C	10.6	2.1	2.1	6.4	0	RT: L100I, K65R PR: N88D	[87]
2002	4 major cities, DR Congo	70	Subset of sentinel survey population representing various subtypes	NA	Multiple	4.3	0	1.4	2.9	0	RT: K103N, V179D PR: M46L, L90M	[96]

Table 2 (continued)

Year	Location	n	Study group	Median CD4 count	Main HIV-1 subtypes	Reported resistance rate (%)*					Mutations	Ref
						Any	NRTI	NNRTI	PI	MDR		
2002	Burundi	101	Selected subset of sentinel serosurvey	NA	C	1.0	0	1.0	0	0	RT: G190E	[97]
2003	Maputo, Mozambique	58	ARV naive subset (DREAM cohort)	361	C	0	0	0	0	0		[81]
2003	Ouagadougou and Bobo Dioulasso, Burkina Faso	97	Recently diagnosed hospital and treatment center attendees (median 33 yrs)	166	CRF02_AG, CRF06_cpx	8.3	2.1	4.1	2.1	0	RT: M41L, T69S, V106A,V108I PR: L33F	[94]
2001-2004	Yaounde, Cameroon	102	Recently diagnosed blood donors and hospital attendees (median 36 yrs)	400	CRF02_AG	7.8	2.9	2.0	2.9	0	RT: A62V, T69N, V108I, M184V, P236L PR: L33F, M46I/L	[94]
2001-2004	Yaounde, Cameroon	96	Pregnant women attending antenatal care, diagnosed <12 months and ARV naive	365	CRF02_AG	2.1	1.0	0	1.0	0	RT: L210W, T215S PR: N88S	[95]
2004	Western Cameroun	54	Antenatal/STD clinic attendees (median 33 yrs) †	NA	CRF02_AG	13.0	3.7	5.6	7.4	3.7	RT: V75I, L100I, M184V, Y188C PR: M46I/L, V82A	[85]
2005-2006	Dar Es Salaam, Tanzania	39	Sentinel serosurvey (WHO Treshold Survey)	NA	A, C	0	0	0	0	0		[90]

Table includes studies published between 2002 and 2007, with a minimum of 20 study participants. Table sorted by year of study. *Resistance subdivided per drug class. †Clonal analysis of proviral analysis, excluding minor populations of drug-resistant mutants from analysis reduces prevalence of any resistance from 13.0% to 1.9%. ARV, antiretroviral; DRC, Democratic Republic of Congo; HIV-1, HIV type-1; MDR, multidrug resistance or resistance to ≥2 classes; NA, not available; NNRTI, non-nucleoside reverse transcriptase inhibitor; NRTI, nucleoside reverse transcriptase inhibitor; PI, protease inhibitor; PR, protease; RT, reverse transcriptase; STD, sexually transmitted disease; WHO, World Health Organization.

Table 3. Summary of studies on rates of NVP resistance in women and infants after exposure to single-dose NVP for pMTCT in sub-Saharan Africa

Year	Location	n	Time (wks) *	Main HIV-1 subtypes	% NVPR women	% NVPR infants	Comments	Ref
NA	Ivory Coast	29	4	CRF02_AG	21%	NA		[118]
NA	South Africa	111+40	4-6	C	67%	53%	2-dose NVP	[117]
NA	South Africa	456	7	C	39%	42%		[114]
NA	South Africa	155+20	26	C	35%	65%	NVPR rate at 6 months of individuals with NVPR at 7 wks. Resistance associated with higher VL and lower CD4 counts	[116]
NA	South Africa	30+30	6	C	40%	40%		[112]
NA	South Africa	68+9	6	NA	57%	78%	Addition of ZDV+3TC to SD-NVP significantly decreased NVPR rates	[115]
1997-2001	Uganda	111+24	6-8	NA	19%	46%	Mutations faded from detection within 12-24 months	[109]
1997-2001	Uganda	279	6-8	A, D	25%	NA	Higher NVPR rates in subtype A (19%) than D (36%)	[111]
1997-2001	Uganda	65	1+6	A, D	28%	NA	Y181C often fades from detection by 6–8 weeks. K103N emerges more slowly, but remains detectable longer	[110]
1997-2001	Uganda	140	1+6	A, D	22%	NA	Y181C fades from detection faster in subtype A and K103N accumulates faster in subtype D	[41]
1997-2003	Uganda, Malawi	306	6-8	A, C, D	69%	NA	Higher NVPR rates in subtype C (69%) than A (19%) or D (36%).	[42]
1997-2003	Uganda, Malawi	41	6-8	A, C, D	na	87%	NVPR more frequent in Malawian subtype C infants (87%) than Ugandan subtype A or D infants (50%)	[44]
2000-2001	Zimbabwe	32	8	C	34%	NA	20 paired breastmilk/plasma samples; NNRTI-resistance in 65% of breastmilk and 50% of plasma RT sequences, with divergent mutation patterns	[113]

Table includes studies using standard genotypic sequencing methods only. Table sorted by country. *Time in weeks after delivery. HIV-1, HIV type-1; NA, not available; NNRTI, non-nucleoside reverse transcriptase inhibitor; NVP, nevirapine; NVPR, nevirapine resistance; pMTCT, prevention of mother-to-child transmission; RT, reverse transcriptase; SD-NVP, single-dose nevirapine; ZDV, zidovudine; 3TC, lamivudine.

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Chapter 3

Multi-nucleoside reverse transcriptase inhibitor resistant HIV type-1 in a patient from Sierra Leone failing stavudine, lamivudine and nevirapine

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ABSTRACT

We report a 33-year-old HIV type-1 (HIV-1)-infected male from Sierra Leone who harboured extensive drug resistance mutations to all nucleoside reverse transcriptase inhibitors (NRTIs) and non-NRTIs, including the multi-NRTI-resistance Q151M complex, K65R, M184I and Y181I, after using standard first-line generic fixed-dose stavudine, lamivudine and nevirapine (Triomune™) for 36 months. In the context of non-B subtypes in resource-limited countries, first-line stavudine-containing regimens have been associated with more extensive and complex mutation patterns, compared with subtype B viruses. Whether the extensive and complex NRTI resistance patterns found among African patients failing first-line antiretroviral therapy is explained by viral genetic diversity or by different patient monitoring strategies remains to be elucidated. Emerging multi-NRTI resistance in sub-Saharan Africa would not only compromise second-line treatment options and the success of antiretroviral rollout, but could also contribute to the spread of drug-resistant variants worldwide.

INTRODUCTION

Expanded access to combination antiretroviral therapy (ART) for HIV type-1 (HIV-1)-infected individuals in sub-Saharan Africa during the past decade [1] has resulted in significant reductions of HIV-1-related morbidity and mortality [2–4]. In resource-limited countries, ART is generally delivered using a public health approach developed by the World Health Organization, which is based on decentralized service delivery, standardized treatment regimens and simplified treatment monitoring [5]. Absence of routine virological monitoring, as is often the reality in poorly resourced settings, might lead to late detection of therapy failure and, consequently, to the continuation of failing regimens after initial virological breakthrough, allowing for accumulation of drug resistance-associated mutations [6]. Moreover, the extensive genetic viral diversity in HIV-1 subtypes and circulating recombinant forms (CRFs) that are present in sub-Saharan Africa have been reported to influence mutational pathways to drug resistance [7], which might have an effect on therapy effectiveness.

CASE PRESENTATION

A 33-year-old HIV-1-infected male from Sierra Leone presented at the outpatient department of the Onze Lieve Vrouwe Gasthuis (OLVG) general hospital (Amsterdam, the Netherlands) with symptoms of nausea, weight loss and painful dark-coloured skin lesions on the foot soles. In Sierra Leone, he had used generic fixed-dose stavudine, lamivudine and nevirapine (Triomune™; Cipla), which is still the most commonly used first-line ART regimen in many sub-Saharan African countries, for 36 months with allegedly good adherence. Recent sputum analysis in Sierra Leone had demonstrated the presence of acid-fast bacilli, suggestive of pulmonary tuberculosis, for which he had used rifampin, isoniazide, pyrazinamide and ethambutol during the past 6 weeks. He presented at the OLVG general hospital during a family visit to the Netherlands and was admitted to the infectious diseases inpatient department for further diagnostic evaluation and treatment. Initial laboratory investigations revealed a low CD4+ T-cell count of 40 cells/μl (pretreatment nadir not documented) and detectable plasma HIV-1 RNA (13,730 copies/ml), indicating treatment failure. Skin biopsy of the foot lesions demonstrated Kaposi's sarcoma. Standard genotypic analysis of the *pol* region was performed using the ViroSeq HIV-1 Genotyping System (Abbott Laboratories, Abbott Park, IL, USA). Detected drug resistance-associated mutations included K65R, V75I, F116Y, Q151M, M184I and Y181I in reverse transcriptase, and multiple resistance-related natural polymorphisms but no major drug resistance-associated mutations in protease. The Y181I mutation confers high-level cross-resistance to all non-nucleoside reverse transcriptase inhibitors (NNRTIs) [8].

HIV-1 variants harbouring Q151M with accompanying mutations V75I and F116Y, known as the multi-nucleoside reverse transcriptase inhibitor (NRTI) resistance Q151M complex, and the K65R resistance mutation, confer extensive cross-resistance to all NRTIs [8]. Phylogenetic analysis using the neighbor-joining method indicated that the sequence belonged to viral clade CRF02_AG. Based on these findings, the antiretroviral regimen was switched to darunavir 600 mg twice daily, ritonavir 100 mg twice daily, raltegravir 400 mg twice daily and enfuvirtide 90 mg twice daily. Because of a subsequent flare of chronic hepatitis B infection (hepatitis B surface antigen-positive, hepatitis B e antigen-negative and HBV DNA 963 IU/ml), possibly related to the withdrawal of lamivudine, tenofovir disoproxil fumarate 245 mg once daily was added to the regimen. *Mycoplasma fortuitum* was cultured in sputum, at which point the tuberculostatic drugs were withdrawn and antibiotic treatment with cotrimoxazole and ofloxacine was initiated. In the course of the following months, the patient showed gradual clinical improvement, with immune restoration, weight gain, regression of Kaposi's sarcoma and complete viral suppression, after which time the ART regimen was simplified.

DISCUSSION

This HIV-1-infected patient from Sierra Leone presented with extensive resistance to all NRTIs after using only a single ART regimen for 3 years. In HIV-1 subtype B viruses, the selection of drug-resistant variants during treatment with stavudine-containing regimens is rather limited. Stavudine usually selects for thymidine analogue mutations (TAMs). Accumulation of ≥2 TAMs is associated with broad NRTI cross-resistance [9, 10]. TAM selection might be reduced or delayed by combination treatments with lamivudine or emtricitabine selecting for the M184V mutation [11]. Q151M usually requires a relatively lengthy period of time to emerge under therapy and has been observed in <5% of HIV-1-infected European patients on long-term NRTI-based ART [12]. K65R and TAMs represent antagonistic pathways of NRTI resistance [13], and K65R is found at low rates among viruses from subtype-B-infected individuals in genotypic databases [14].

In the context of non-B subtypes in resource-limited countries, however, first-line stavudine-containing regimens have been associated with more extensive, less predictable and more complex mutation patterns. Several recent studies in subtype-C-infected patients who experienced treatment failure have reported considerable rates of NRTI mutations including K65R [15–19] and Q151M [16, 18]. Notably, a recent study reported the presence of extensive NRTI resistance in a subtype-C-infected Malawian cohort after clinical or immunological failure on fixed-dose stavudine, lamivudine and nevirapine, with 56% TAMs, 23% K70E or K65R, 19% Q151M and 16% Q151M associated with either

K65R or K70E [16]. It was recently suggested that the presence of two subtype-C-specific nucleotide polymorphisms at positions 64 and 65 in reverse transcriptase could favour the selection of K65R [20]. In patients infected with CRF02_AG who fail stavudine-containing regimens, the multi-NRTI resistance Q151M complex has been anecdotally reported [21], and has been associated with K65R [15]. Additional studies are warranted to establish whether certain NRTI resistance mutations (particularly K65R and/or Q151M) are preferentially selected in the various non-B subtypes.

Currently, there is no robust evidence that the possible added survival benefit of routine viral load monitoring in resource-limited settings is cost-effective. A recent review demonstrated, however, that genotypic resistance to lamivudine, NRTIs (TAMs), and NNRTIs appeared substantially higher in less frequently virologically monitored patients who experienced treatment failure, compared with frequently monitored patients [6]; therefore, the potential long-term impact of inadequately guided treatment changes on resistance, subsequent treatment outcomes and the spread of resistance among the wider population should receive more attention.

Given that ART is a lifelong intervention and that roll-out programmes in sub-Saharan African countries mature, increasing numbers of patients are expected to fail their first-line regimens, requiring switch to second-line regimens [22]. Current standard second-line regimens, if available, combine a ritonavir-boosted protease inhibitor (bPI) with two previously unused and/or recycled NRTIs [5]. Once multi-NRTI resistance has occurred, standard second-line regimens will primarily offer the benefit of the bPI, with limited or no additional effect of the NRTI backbone. Data available to date suggest that mono-bPI therapy might be clinically successful, but the selection of resistance to bPIs in PI-naive individuals has been reported after such therapy [23–25].

In conclusion, further research is warranted to elucidate whether the extensive and complex NRTI resistance patterns found among African patients failing first-line ART is explained by viral genetic diversity or different patient monitoring strategies. Emerging multi-NRTI-resistance in sub-Saharan Africa would not only compromise second-line treatment options and the success of antiretroviral rollout, but could also contribute to the spread of drug-resistant variants worldwide. Strategies should be directed at minimizing accumulation of drug resistance by developing cost-effective laboratory monitoring strategies, phasing out the use of stavudine, and enhancing access to simple and robust second-line options with non-overlapping drug resistance profiles.

Contributors

RLH, MSA and JPHF were involved in the clinical management of the patient and conceived the idea for this report. NKTB performed the genotypic resistance test. RLH wrote the first draft of the manuscript. AMJW and JPHF revised it critically for important intellectual content. All authors contributed to subsequent drafts and reviewed and approved the final manuscript.

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Chapter 4

Cohort Profile: the PharmAccess African (PASER-M) and the TREAT Asia (TASER-M) Monitoring Studies to Evaluate Resistance – HIV drug resistance in sub-Saharan Africa and the Asia-Pacific

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HOW DID THE STUDY COME ABOUT?

According to World Health Organization (WHO) estimates, 33.4 million people were infected with the human immunodeficiency virus (HIV) type 1 globally at the end of 2008 [1]. Sub-Saharan Africa and Asia are the two regions having the highest HIV prevalence with 22.4 million and 4.7 million people infected, respectively [1]. During the 5 years prior, access to combination antiretroviral therapy (ART) in low- and middle-income countries increased 10-fold to reach 4 million people, providing coverage to 28% of those in need [2]. Several studies have reported significant reductions in HIV-related morbidity and mortality for individuals with access to treatment in these regions [3-5]. In resource-limited settings, to facilitate the rapid expansion of access to ART, WHO recommends a standardized, public health approach [6]. This is in contrast to the individualized patient-management strategies in developed countries, based on routinely available diagnostic monitoring [7]. Standardized first-line ART regimens consist of a non-nucleoside reverse transcriptase inhibitor (NNRTI) and a dual nucleoside/nucleotide reverse transcriptase inhibitor (NRTI) backbone, available in some countries as generic fixed-dose combinations [6]. Recommended second-line regimens combine a ritonavir-boosted protease inhibitor (PI) with two previously unused and/or recycled NRTIs [6].

Routine HIV viral load monitoring is not generally available in resource-limited countries and treatment failure is frequently identified based on immunological definitions and/or the occurrence of clinical events [6]. Virological breakthrough may be detected late while the failing regimen is continued, thus facilitating the acquisition and accumulation of drug resistance-associated mutations [8]. Drug-resistant HIV variants may compromise the effectiveness of subsequent lines of treatment and their transmission to newly infected individuals has severe public health consequences [9, 10]. To date, ART programmes have been implemented without accompanying HIV drug resistance (HIVDR) monitoring. Monitoring studies are hampered by the lack of a molecular laboratory infrastructure required for genotypic resistance testing, logistical challenges related to maintaining specimen integrity in remote settings and the high costs of testing [11]. Challenges to scaling up ART in resource-limited countries, such as absence of routine virological monitoring and limited choices of drug regimens, advocate for the development of a global public-health framework to monitor and prevent the emergence of HIVDR and thus maximize long-term ART effectiveness [12].

HIV-1 subtype B is the predominant viral subtype in North America, Western Europe and Australia, and antiretroviral (ARV) drugs have been developed on this subtype. However, in sub-Saharan Africa and Asia, the genetic diversity in HIV subtypes and circulating recombinant forms (CRFs), resulting from recombination between subtypes

within a dually infected person, is extensive [13]. Although current evidence is limited, some reports have suggested that the propensity to develop HIVDR and the spectrum of mutations that emerge during drug selective pressure, may differ across subtypes and CRFs [14-17]. Viral heterogeneity may, therefore, have implications for rates of disease progression and patient response to ART, warranting further study of inter-subtype differences in mutational pathways to resistance.

To help assess the extent of HIVDR in sub-Saharan Africa and Asia, a collaborative bi-regional programme was established, called LAASER [Linking African and Asian Societies for an Enhanced Response to HIV/AIDS; http://www.laaser-hivaids.org] with the primary aim of increasing regional capacities for the monitoring of HIVDR. PharmAccess Foundation, a non-profit organization dedicated to the strengthening of health systems and improving access to quality basic health care in sub-Saharan Africa, has developed the PharmAccess African Studies to Evaluate Resistance (PASER). TREAT Asia (Therapeutics, Research, Education and AIDS Training in Asia) is a network of clinics, hospitals and research institutions working to ensure safe and effective delivery of HIV/AIDS treatment throughout the Asia-Pacific and has developed the TREAT Asia Studies to Evaluate Resistance (TASER). Both PASER and TASER programmes incorporate a monitoring and evaluation (M) and a surveillance (S) protocol. Laboratories providing genotyping results for PASER and TASER are required to participate in the TREAT Asia Quality Assurance Scheme (TAQAS) which is an ongoing assessment program to build genotyping laboratory capacity, described elsewhere [18]. The focus of this cohort profile is the monitoring and evaluation protocols, PASER-M and TASER-M.

HOW ARE PASER AND TASER SET UP AND HOW ARE THEY FUNDED?

Through the LAASER program, PASER and TASER receive financial support from the Dutch Ministry of Foreign Affairs through a partnership with Stichting Aids Fonds, PharmAccess Foundation, TREAT Asia (a programme of amfAR, The Foundation for AIDS research) and International Civil Society Support. PASER-M is coordinated by PharmAccess Foundation, in collaboration with the Amsterdam Institute for Global Health and Development (AIGHD) and the Virology Department at the University Medical Center Utrecht, The Netherlands. TASER-M is coordinated by TREAT Asia and its statistical and data management centre is the National Centre in HIV Epidemiology and Clinical Research (NCHECR), The University of New South Wales in Sydney, Australia.

PASER constitutes a newly established collaboration between HIV treatment clinics, laboratories with the capacity to perform genotypic sequencing and research centers.

Thirteen clinical sites and two reference laboratories in six African countries (Kenya, Nigeria, South Africa, Uganda, Zambia and Zimbabwe) are collaborating on PASER-M (figure 1a). Details of the PASER-M collaborating clinical sites are summarized in table 1. Ethics approvals were obtained from the Academic Medical Center Institutional Review Board (IRB) and local IRBs. Sites are government, non-government, faith-based or private clinics and hospitals, situated in major cities or rural areas. ART was introduced at the sites at varying time points between 1992 and 2006 (median: 2004). Of the 13 sites, 11 provided drugs, consultations and routine lab testing free of charge. HIV viral load testing is available at 8 of 13 sites.

TASER collaborating sites are selected from within the existing TREAT Asia network based on their laboratory capacity to perform genotypic sequencing, described elsewhere [18, 19]. Sites that do not have internal laboratory genotyping capacity can participate through collaboration with a TAQAS-certified laboratory. Eleven clinical and laboratory sites in six Asian countries (China, Indonesia, Malaysia, Philippines, South Korea, Thailand) are collaborating on TASER-M (figure 1b). Ethics approvals were obtained from local IRBs having Federal wide Assurances (FWAs) in place from the United States Office for Human Research Protections. FWAs are required for TASER sites as they participate in the International Epidemiologic Databases to Evaluate AIDS (IeDEA) initiative, described elsewhere [20]. Sites are generally government or university-based clinics and hospitals or private clinics, situated in major cities and other urban areas. Those with ethics approvals prior to June 2010 are shown in figure 1b. ART has been available in Asia for more than 10 years, even in less-resourced countries in the region and all TASER-M clinical sites have on-site viral load testing.

Clinical sites follow their national guidelines to assess eligibility for ART initiation in accordance with the WHO recommendations [6]. Genotypic resistance testing on PASER and TASER clinical specimens are performed in TAQAS-certified genotyping laboratories [18]. Laboratories are encouraged to become accredited members of the WHO/HIVResNet HIV Drug Resistance Laboratory network [21]. Population-based nucleotide sequencing of the HIV protease (PR) and partial reverse transcriptase (RT) gene regions is performed on plasma specimens, which have HIV RNA of more than 1000 copies/ml. Plasma is obtained from blood collected in EDTA-tubes which is locally stored at -80°C and, if required, batch-shipped on dry ice to a genotyping laboratory.

PASER-M genotypic testing is concentrated in two central reference laboratories, and thus depends on a robust cold-chain and web-based specimen tracking system for managing specimen shipments. Approximately half of TASER-M clinical sites have an on-site or local genotyping laboratory. Most genotyping laboratories amplify viral

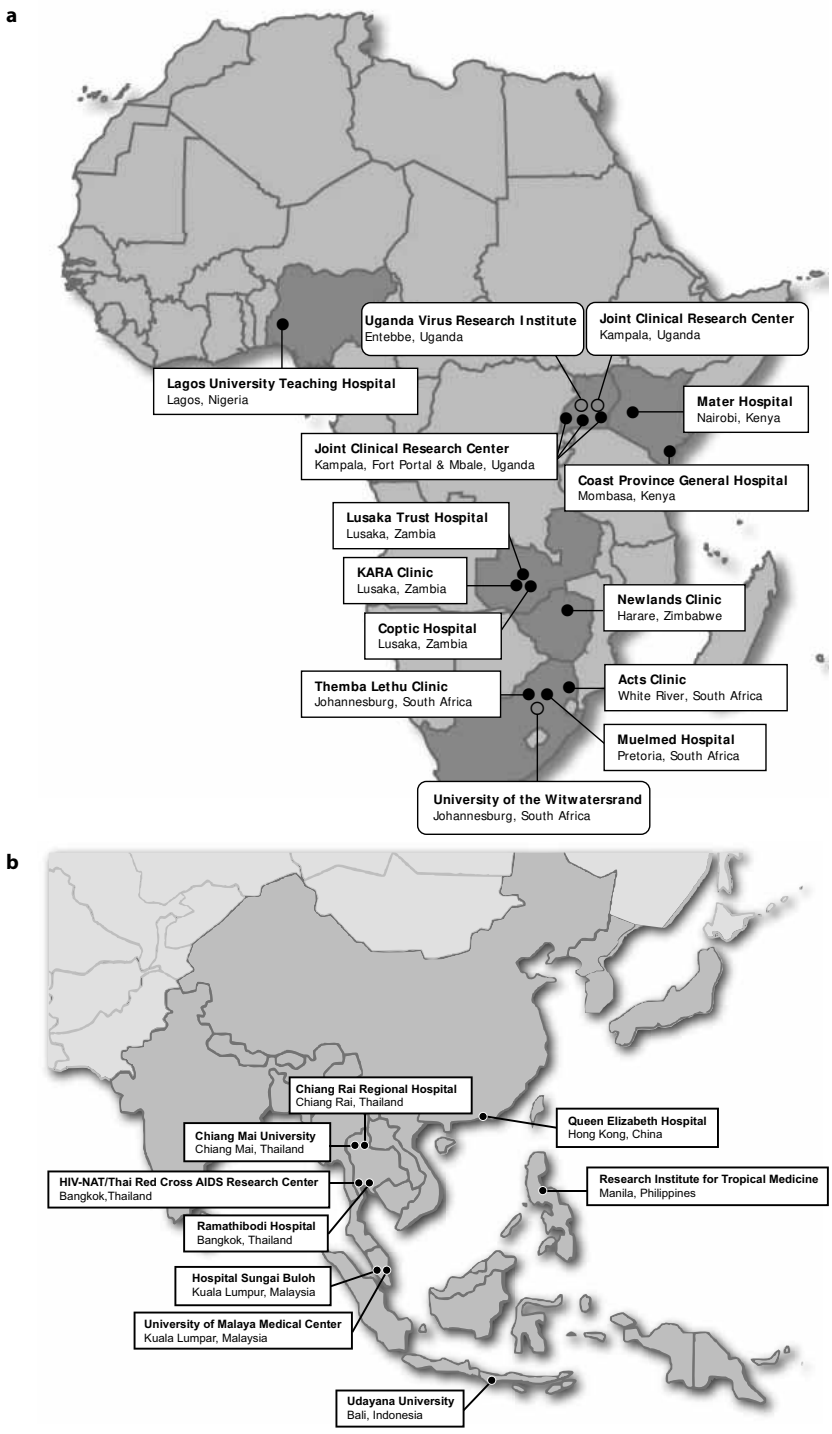

Figure 1. Geographical location of (a) PASER-M collaborating sites and (b) TASER-M collaborating sites

Table 1. Characteristics of the PASER-M collaborating clinical sites [a]

Country	Site	Sector	Site type	Setting	Year of ART introduction	Total adults HIV+	Total adults on ART	Research experienced	Number of staff [b]	Number of patients per staff [b]	Free care [c]	Patient tracing [e]	HIV viral load available	Main funding source	Month/ year of study initiation
Zambia	ZA/LTH	PFP	General hospital	Urban	1997	675	625	No	11	61	No [d]	No	Yes	Clients	03/07
	ZA/KAR	NGO	HIV clinic	Urban	2004	1540	870	No	7	220	Yes	Yes	No	CIDRZ/PEPFAR	03/07
	ZA/CHC	FBO	General hospital	Urban	2006	883	494	No	4.5	196	Yes	Yes	No	CHAZ/PEPFAR	05/07
S Africa	SA/MMH	PFP	HIV clinic	Urban	2000	2300	1150	Yes	3	767	No [d]	Yes	Yes	Clients	05/07
	SA/TLC	Public	HIV clinic	Urban	2004	14000	9500	Yes	15	933	Yes	Yes	Yes	MOH/USAID	09/07
	SA/ACC	NGO	HIV clinic	Rural	2004	4126	1201	No	5	825	Yes	Yes	Yes	USAID/PEPFAR	11/07
Zimbabwe	ZI/CON	NGO	HIV clinic	Urban	2004	1521	1292	Yes	14	109	Yes	Yes	No	SACI/SDC	09/08
Uganda	UG/JCR	Public	HIV clinic	Urban	1992	8308	4871	Yes	62	134	Yes	Yes	Yes	USAID/PEPFAR	01/08
	UG/JFP	Public	HIV clinic	Rural	2003	4849	2502	No	7	693	Yes	Yes	Yes	USAID/PEPFAR	01/08
	UG/MBA	Public	HIV clinic	Rural	2002	3615	2823	No	6	603	Yes	Yes	Yes	USAID/PEPFAR	02/08
Kenya	KE/CRH	Public	General hospital	Urban	2003	10653	4788	Yes	17	627	Yes	Yes	No	MOH/USAID	11/07
	KE/MAT	FBO	General hospital	Urban	2006	1402	839	No	7	200	Yes	Yes	No	PEPFAR	02/08
Nigeria	NI/LUT	Public	Teaching hospital	Urban	2002	5507	4237	Yes	16	344	Yes	Yes	Yes	MOH/PEPFAR	09/08

ART, combination antiretroviral therapy; NGO, non-governmental organization; FBO, faith-based organization; PFP, private for-profit; CIDRZ, Center for Infectious Diseases Research Zambia; PEPFAR, President's Emergency Plan for Aids Relief; CHAZ, Churches' Health Association Zambia; USAID, United States Agency for International Development; SACI, Swiss Aids Care International; SDC, Swiss Agency for Development and Cooperation; MOH, Ministry of Health;

[a] Data reflect situation at time of study initiation

[b] Medical doctors, clinical officers and nurses

[c] Includes consultations, medication and routine laboratory tests

[d] Approximation of cost (€) for consultation, ART/month, CD4 and HIV RNA test: SA/MMH 23, 28-46, 11, 59, respectively; ZA/LTH 10, 67-200, 27, 60, respectively

[e] Mostly by means of telephone calls to no-show patients

sequences using in-house methods, based on assembled commercially available assay components and laboratory-specific sequencing and amplification primers. One TASER laboratory uses the commercial kit TruGene (Bayer HealthCare, Tarrytown, NY, USA). The online Stanford interpretation system is used by most laboratories to identify drug resistance-associated mutations [22]. Resistance genotyping is generally performed retrospectively (i.e. not real-time) for all participants. Details of the genotyping laboratories are summarized in table 2. PASER and TASER sequences are submitted to the ViroScore database (Advanced Biological Laboratories SA, France) for data storage.

Table 2. PASER and TASER Genotyping Laboratories

Country	Genotyping laboratory	Sequencing Technology	Editing Software	Regions sequenced	Interpretation System
China	Hong Kong AIDS Trust Fund Molecular Laboratory, Dept of Microbiology, Queen Mary Hospital, Hong Kong [a]	In-house assay, BigDye Terminator, ABI	Staden Package version 1.6.0	PR: 1-99; RT: 1-400	Stanford
Malaysia	University of Malaya, HIV Research Laboratory, Medical Microbiology Department, University of Malaya, Kuala Lumpur [a]	In-house assay, PRISM® 3100-Avant Genetic Analyzer, ABI	BioEdit Version 7.0.9.0, Chromas Lite version 2.01	PR: 1-99; RT: 1-400	Stanford
South Africa	Department of Molecular Medicine and Haematology, University of the Witwatersrand, Johannesburg [b]	In-house assay, Prism 3100-*Avant* and 3730 Genetic Analyzer, ABI	Sequencher version 4.5	PR: 1-99; RT: 1-230	Stanford
Thailand	Vaccine and Cellular Immunology Laboratory (VCI lab), Division of Allergy and Clinical Immunology, Department of Medicine, Faculty of Medicine, Chulalongkorn University, Bangkok	In-house assay, BigDye Terminator, ABI	Sequence Navigator, ABI	PR: 1-99; RT: 20-270	Stanford
	Virology and Molecular Microbiology Unit, Ramathibodi Hospital, Mahidol University, Bangkok	TruGene, Bayer HealthCare	TruGene version 11	PR: 4-99; RT: 36-247	TruGene version 12
	HIV-1 Genotyping Laboratory, Chiang Mai University, Chiang Mai [a]	In-house assay, BigDye Terminator, ABI	SeqScape v2.0, ABI	PR: 1-99; RT: 1-250	Stanford
Uganda	Joint Clinical Research Center, Kampala [c]	In-house assay, CEQ 8000 Analyzer, BC	BioEdit version 7.0.9.0	PR:1-99; RT: 1-300	Stanford

[a] Laboratories serving 2 TASER-M clinical sites;

[b] Laboratory serving 7 PASER-M sites in southern Africa;

[c] Laboratory serving 6 PASER-M sites in east and west Africa

Manufacturer details: Applied Biosystems, Foster City, CA, USA; Bayer HealthCare, Tarrytown, NY, USA; Beckman Coulter Inc., Fullerton, CA, USA; BioEdit, www.mbio.ncsu.edu/BioEdit/bioedit.html; Chromas Lite, Technelysium, Queensland, Australia; Genecodes, Ann Arbor, MI, USA; Staden Package, http://staden.sourceforge.net/

WHAT DO PASER-M AND TASER-M COVER AND WHO IS INCLUDED IN THE SAMPLE?

The monitoring studies are multi-center prospective cohort designs with sequential patient enrolment. Patient eligibility criteria are listed in table 3. The main study objectives are to assess prevalence and incidence of HIVDR, mutational patterns and factors associated with HIVDR in persons initiating first-line ART or switching to a second-line regimen due to treatment failure under routine circumstances. Participants are required to sign informed consent prior to study enrolment and must initiate or switch ART within 30 days (PASER-M) or 181 days (TASER-M) following baseline specimen collection. Regimen switch due to treatment failure may be determined clinically, as assessed by disease progression, immunologically, by CD4 cell count, or virologically, by HIV viral load. A single drug substitution, due to toxicity or intolerance, is not considered a regimen switch. Each site aims to recruit a total of 240 participants. Second-line participants are recruited among first-line participants failing therapy and the clinical site patient population. The recommended maximum site-specific enrolment period is 18 months.

Table 3. Patient eligibility criteria for PASER-M and TASER-M

Inclusion criteria
Confirmed HIV-1 infection
≥18 years of age
Eligible [a] for initiation of a first-line ART regimen, or switch from a first-line ART regimen (containing at least three antiretroviral drugs and taken for at least six months) to a second-line ART regimen due to virological, immunological and/or clinical failure
Signed informed consent for study participation prior to enrolment
Exclusion criteria
Currently taking ART (minimum of 3 drug regimen), if initiating a first-line ART regimen [b]
Pregnancy at enrolment [c]
HIV-1/2 dual-infection (in endemic countries only) [c]

ART, combination antiretroviral therapy

[a] Eligibility for ART initiation defined in accordance with national ART guidelines (i.e. advanced immunodeficiency as defined by CD4 cell count <200 or <350 cells/µl, or advanced clinical disease according to WHO clinical stage/CDC classification).

[b] Specified PASER-M definition: re-initiation of a first-line ART regimen < 30 days after stopping previous first-line ART (previous use of antiretroviral prophylaxis or mono/dual therapy is not an exclusion criterion).

[c] Exclusion criteria applicable to PASER-M only

HOW OFTEN ARE PARTICIPANTS FOLLOWED-UP? WHAT DATA ARE BEING COLLECTED?

Participants are followed up as per local standard of care guidelines. The frequency of follow-up visits for patients varies by site (range: every 1 to 6 months). The studies make

use of clinical data collected during routine visits and recorded in medical records. HIV viral load measurement and, if HIV RNA value is more than 1000 copies/ml, genotypic resistance testing is performed on plasma specimens taken at baseline, prior to regimen switch due to treatment failure and at annual follow-up. For patients failing a first-line regimen, the treatment failure data collection becomes the new baseline for the second-line regimen. Annual follow-up is then calculated from this point. For patients failing a second-line ART, the treatment failure data collection is the final assessment prior to the patient going off study.

PASER-M clinical data are recorded on standardized hard-copy data forms which are completed at three-monthly intervals and entered in a web-based clinical data system, called the HAART Monitoring System. PharmAccess performs quality assurance measures which include (i) source data verification during 3 to 6-monthly site audits, (ii) checks to identify data entry inconsistencies or suspect data values and (iii) specimen tracking. TASER-M site personnel extract clinical data from site databases and medical records collected as part of usual care. From March 2008 to March 2009, TASER-M data were submitted electronically to NCHECR on a quarterly basis, as part of study start up, then at 6 monthly intervals. At each transfer, NCHECR performs quality assurance measures which include (i) checks to identify data entry inconsistencies or suspect data values, (ii) specimen tracking and (iii) ARV history completeness. Annually, a random 10% of TASER-M patients are selected for internal site audit where submitted data is compared to patient medical records.

The studies capture standardized virologic and genotypic data at protocol determined intervals. Genotyping data consists of HIV subtype and HIVDR mutations, including insertions and deletions. TASER-M also records discordant subtypes, i.e. when the PR and RT region subtypes differ. Laboratory specimen tracking information is recorded during specimen processing, allowing assessment of pre-analytical and assay validity. Genotyping laboratories complete an annual laboratory survey which includes the dynamic range of the virologic assay used, the regions of PR and RT genome routinely sequenced and the interpretation algorithm used. Observational patient data includes demographic parameters, physical measures, Centers for Disease Control and Prevention (CDC) class (TASER-M) or WHO clinical stage (PASER-M), serology of hepatitis and syphilis (TASER-M), opportunistic infections, current ART regimen, ARV history, concomitant medications, routine laboratory parameters (including CD4 counts) and assessment of drug adherence. Main analyses will include age, sex, ethnicity, HIV exposure category, WHO clinical stage (PASER-M) or CDC class (TASER-M), viral hepatitis co-infection status, CD4 count, HIV viral load, HIV subtype, drug adherence, ARV history and ART regimen as covariates. Predictors of drug resistance will be assessed using logistic regression models. Incidence

of drug resistance will be summarized using person-years methods and Kaplan-Meier plots. Cox proportional hazards models will be used to assess risk factors associated with developing drug resistance.

WHAT IS THE ANTICIPATED ATTRITION?

The actual attrition in PASER-M and TASER-M cannot currently be accurately estimated because the duration of follow-up in the databases is still limited. In sub-Saharan Africa patient retention in routine ART programmes has been estimated at 61.6% [23] to 66.8% [2] at 24 months on ART, attrition being mainly due to loss of follow-up and early death [23]. Therefore, in PASER-M the original site-specific sample size was calculated accounting for 20% loss to follow up and 25% mortality after 24 months. Attrition is expected to vary between sites, as a result of differences in patient populations, care provided and provisions for tracing lost to follow-up. TASER-M sites are generally sourced from the ongoing TREAT Asia HIV Observational Database (TAHOD) [19]. Loss to follow-up for TAHOD was 6.9/100 person-years for the 12-month period from September 2007 to September 2008. Since TASER-M monitors specified outcomes, we speculate that TASER-M follow-up will be similar to TAHOD or better.

WHAT HAS BEEN FOUND?

PASER-M

Patient recruitment commenced in March 2007 and was completed in September 2009. Of the 13 sites, 12 reached the site-specific target of 240 participants, enrolling a total of 3005 participants. Excluding patients with protocol violations (n=16) and key data missing (n=4), 2985 patients were included in the analysis. Of these, 2736 (91.6%) were eligible for a first-line ART regimen and 249 (8.3%) were eligible for second-line ART due to treatment failure. Patient characteristics are summarized in table 4. For first-line patients, the median age was 36.8 years (interquartile range, IQR 31.3-42.6) and 58% were women. HIV exposure was predominantly reported as heterosexual contact. More than 60% had advanced disease (classified as WHO stage III or IV) and 37% had pre-therapy CD4 counts of less than 100 cells/μL. Across all 13 sites, median baseline CD4 counts of first-line patients were less than 200 cells/μl (site median 135 cells/μL, range: 93 – 191). Median baseline HIV viral load was 4.9 $\log_{10}$ copies/ml (IQR 4.2-5.5). The most frequently prescribed first-line regimens were based on NNRTIs (99.7%), i.e. efavirenz (EFV) and nevirapine (NVP) at 60% and 40%, respectively. First-line dual NRTI backbones were predominantly lamivudine (3TC)/zidovudine (AZT) (37%), emtricitabine (FTC)/

Table 4. Baseline patient characteristics, by region and line of ART

	PASER-M (Africa)				TASER-M (Asia)		
	Total	Initiation of first-line ART [a]		Switch to second-line ART [b]	Initiation of first-line ART [a]		Switch to second-line ART [b]
		ARV-naive	ARV-experienced [c]		ARV-naive	ARV-experienced [c]	
Patients, no. (%)	3713	2598 (87.0)	138 (4.6)	249 (8.3)	693 (95.2)	10 (1.4)	25 (3.4)
Sex							
Female – no. (%)	1988 (53.5)	1494 (57.5)	105 (76.1)	124 (49.8)	239 (34.5)	10 (100.0)	16 (64.0)
Male – no. (%)	1725 (46.5)	1104 (42.5)	33 (23.9)	125 (50.2)	454 (65.5)	0 (0.0)	9 (36.0)
Age (years), median (IQR)	36.9 (31.7-43.3)	37.0 (31.2-42.8)	34.7 (29.2-40.2)	38.6 (32.9-44.2)	36.5 (31.1-43.2)	33.1 (27.4-38.4)	36.5 (32.4-41.9)
18-29	707 (19.0)	490 (18.9)	39 (28.3)	28 (11.2)	143 (20.6)	3 (30.0)	4 (16.0)
30-39	1668 (44.9)	1176 (45.3)	65 (47.1)	112 (45.0)	298 (43.0)	5 (50.0)	12 (48.0)
≥40	1338 (36.0)	932 (35.9)	34 (24.6)	109 (43.8)	252 (36.4)	2 (20.0)	9 (36.0)
HIV exposure, no. (%)							
Heterosexual contact	2583 (69.6)	1731 (66.6)	108 (78.3)	191 (76.7)	520 (75.0)	10 (100.0)	23 (92.0)
Homosexual contact	134 (3.6)	4 (0.2)	0 (0.0)	0 (0.0)	128 (18.5)	0 (0.0)	2 (8.0)
Other [d]	996 (26.8)	863 (33.2)	30 (21.7)	58 (23.3)	45 (6.5)	0 (0.0)	0 (0.0)
WHO clinical stage, no. (%)							
I	493 (16.5)	393 (15.1)	25 (18.1)	75 (30.1)	na	na	na
II	711 (23.8)	623 (24.0)	33 (23.9)	55 (22.1)	na	na	na
III	1281 (42.9)	1145 (44.1)	59 (42.8)	77 (30.9)	na	na	na
IV	500 (16.7)	437 (16.8)	21 (15.2)	42 (16.9)	na	na	na
CDC classification, no. (%)							
A	302 (41.5)	na	na	na	296 (42.7)	0 (0.0)	6 (24.0)
B	166 (22.8)	na	na	na	152 (21.9)	10 (100.0)	4 (16.0)

Table 4 (continued)

	PASER-M (Africa)				TASER-M (Asia)		
	Total	Initiation of first-line ART [a]		Switch to second-line ART [b]	Initiation of first-line ART [a]		Switch to second-line ART [b]
		ARV-naive	ARV-experienced [c]		ARV-naive	ARV-experienced [c]	
C	260 (35.7)	na	na	na	245 (35.4)	0 (0.0)	15 (60.0)
Ever pulmonary tuberculosis, no. (%)	741 (20.0)	569 (21.9)	25 (18.1)	74 (29.7)	69 (10.0)	na	4 (16.0)
Hepatitis B [f] – no. (%)	36 (4.9)	na	na	na	35 (5.1)	0 (0.0)	1 (4.0)
Hepatitis C [g] – no. (%)	56 (7.7)	na	na	na	55 (7.9)	0 (0.0)	1 (4.0)
History of ARV drug use, no. (%)	422 (11.4)	na	138 (100.0)	249 (100.0)	na	10 (100.0)	25 (100.0)
ART	334 (9.0)	na	60 (43.5)	249 (100.0)	na	0 (0.0)	25 (100.0)
Mono or dual therapy	10 (0.3)	na	6 (4.3)	4 (1.6)	na	0 (0.0)	0 (0.0)
Single-dose NVP for PMTCT	36 (1.0)	na	35 (25.4)	1 (0.4)	na	0 (0.0)	0 (0.0)
Combination therapy for PMTCT	31 (0.8)	na	19 (13.8)	2 (0.8)	na	10 (100.0)	0 (0.0)
Unspecified	22 (0.6)	na	22 (15.9)	0 (0.0)	na	0 (0.0)	0 (0.0)
CD4 cell count (cells/μL), median (IQR)	129 (56-205)	133 (62-204)	177 (92-262)	125 (46-196)	99 (33.5-201)	169 (151-222)	197 (109-299)
<100	1456 (39.2)	975 (37.5)	38 (27.5)	102 (41.0)	337 (48.6)	1 (10.0)	3 (12.0)
100-199	1215 (32.7)	914 (35.2)	42 (30.4)	81 (32.5)	164 (23.7)	6 (60.0)	8 (32.0)
≥200	1007 (27.1)	702 (27.0)	57 (41.3)	64 (25.7)	171 (24.7)	3 (30.0)	10 (40.0)
Unknown	25 (0.7)	7 (0.3)	1 (0.7)	2 (0.8)	21 (3.0)	0 (0.0)	4 (16.0)
HIV RNA (log_{10} c/ml), median (IQR)	4.9 (4.3-5.5)	4.9 (4.3-5.6)	4.8 (4.2-5.5)	4.1 (3.2-5.0)	5.0 (5.4-6.8)	4.8 (4.5-5.0)	4.0 (3.6-4.5)
ART regimen							
NNRTI-based triple regimen	3330 (89.7)	2590 (99.7)	135 (97.8)	2 (0.8)	593 (85.6)	10 (100.0)	0 (0.0)

Table 4 (continued)

	PASER-M (Africa)				TASER-M (Asia)		
	Total	Initiation of first-line ART [a]		Switch to second-line ART [b]	Initiation of first-line ART [a]		Switch to second-line ART [b]
		ARV-naive	ARV-experienced [c]		ARV-naive	ARV-experienced [c]	
AZT-containing	1302 (35.1)	964 (37.2)	51 (37.8)	1 (0.4)	170 (28.7)	10 (100.0)	0 (0.0)
TDF-containing	1088 (29.3)	868 (33.5)	49 (36.3)	1 (0.4)	110 (18.5)	0 (0.0)	0 (0.0)
d4T-containing	833 (22.4)	690 (26.6)	33 (24.4)	0 (0.0)	276 (46.5)	0 (0.0)	0 (0.0)
ABC-containing	106 (2.9)	68 (2.6)	2 (1.5)	0 (0.0)	37 (6.2)	0 (0.0)	0 (0.0)
3TC-containing	2387 (71.7)	1746 (67.4)	89 (65.9)	0 (0.0)	542 (91.4)	10 (100.0)	0 (0.0)
FTC-containing	942 (28.3)	843 (32.5)	48 (35.6)	0 (0.0)	49 (8.3)	0 (0.0)	0 (0.0)
PI-based triple regimen	351 (9.5)	6 (0.2)	0 (0.0)	247 (99.2)	73 (10.5)	0 (0.0)	25 (100.0)
Triple NRTI regimen	29 (0.8)	2 (0.1)	3 (2.2)	0 (0.0)	24 (3.5)	0 (0.0)	0 (0.0)
NNRTI+PI-based regimen	3 (0.1)	0 (0.0)	0 (0.0	0 (0.0)	3 (0.4)	0 (0.0)	0 (0.0)

Data are n (%) of patients, unless otherwise indicated. na, not available; ART, combination antiretroviral therapy; ARV, antiretroviral; CDC, US Center for Disease Control and Prevention; WHO, World Health Organization; PMTCT, prevention of mother-to-child transmission of HIV-1; IQR, interquartile range; NVP, nevirapine; d4T, stavudine; AZT, zidovudine; TDF, tenofovir; ABC, abacavir; 3TC, lamivudine; FTC, emtricitabine; NRTI, nucleoside reverse transcriptase inhibitor; NNRTI, non-nucleoside reverse transcriptase inhibitor; PI, protease inhibitor

[a] Eligibility for ART initiation in accordance with national ART guidelines (i.e. advanced immunodeficiency as defined by CD4 cell count <200 or <350 cells/µl, or advanced clinical disease according to WHO clinical stages or CDC classification).

[b] Regimen switch due to treatment failure, defined by local standard of care guidelines as determined clinically, immunologically or virologically.

[c] ARV-experienced is defined as any previous use of ARVs, i.e. (first-line) ART, mono/dual therapy, and/or PMTCT

[d] Includes recipients of blood products, injecting drug users, perinatal transmission and unknown exposures

[f] Hepatitis B positive status was defined as being HBsAg positive

[g] Hepatitis C positive status was defined as being HCV antibody positive

tenofovir (TDF) (34%) and 3TC/stavudine (d4T) (26%). Overall, 67% of patients started a 3TC-containing first-line regimen. Among patients initiating first-line ART, 95% (n=2 598) reported to be ARV-naïve and 5% (n=138) had previous ARV experience, which included ART (n=60), mono/dual therapy (n=6), single-dose NVP for prevention of mother-to-child transmission of HIV (PMTCT) (n=35), combination therapy for PMTCT (n=19), and unspecified (n=22). Compared with ARV-naive first-line patients, ARV-experienced first-line patients had higher median CD4 counts (177 vs. 133 cells/μL, p<0.0001), were younger (median 34.7 vs. 37.0 years, p<0.0001) and were more likely to be female (76.1% vs. 57.5%; p<0.001). Other baseline characteristics did not differ between ARV-naïve and ARV-experienced patients.

For the 249 (8.3%) patients switching to second-line ART, the median age was 38.6 years (IQR: 32.9-44.2) and sex was equally distributed. HIV exposure was predominantly heterosexual contact and 48% of patients had advanced disease (classified as WHO stage III or IV). Median CD4 count was 125 cells/μL (IQR 46-196). Median pre-switch HIV viral load was 4.1 $\log_{10}$ copies/ml (IQR 3.2-5.0). Ritonavir-boosted lopinavir (LPV) was the PI used almost exclusively (98%).

As shown in table 5, analysis of the first available 1795 viral sequences demonstrated that the most common HIV subtypes in the cohort were C (1216, 68.7%), A (338, 18.7%) and D (179, 10.0%). The first PASER report published in 2008 reviewed the available data on HIVDR in sub-Saharan Africa [11]. Baseline HIVDR data from Lusaka, Zambia has recently been published [24]. International presentations have summarized preliminary baseline HIVDR mutations and subtype distributions [25, 26].

TASER-M

Patient recruitment commenced in March 2007 and for the March 2009 transfer, seven sites from Thailand, Hong Kong and Malaysia provided data. Of 773 patients, 755 (97.7%) commenced ART within 181 days of baseline specimen collection and 728 (96.4%) participants had genotypic data available. Of these, 693 (95.2%) ARV-naïve patients and 10 (1.4%) ARV-experienced patients, following prior PMTCT, were eligible for first-line regimens. A further 25 (3.5%) patients were eligible for second-line ART following first-line treatment failure. Patient characteristics are summarized in table 4. For ARV-naïve first-line patients, the median age was 36.5 years. Almost two-thirds of patients were male and HIV exposure was predominantly heterosexual contact. More than one third of patients were classified as CDC class C and almost half of patients had pre-therapy CD4 counts less than 100 cells/μL. Median baseline HIV viral load was 5.0 $\log_{10}$ copies/ml (IQR 5.4-6.8) and the most common first-line regimens were based on NNRTIs (85.6%). Excluding 14 (2.4%) patients on a randomized clinical trial with a blinded NNRTI com-

Table 5. HIV subtypes and circulating recombinant forms by region and country

	Total	PASER-M (Africa)						TASER-M (Asia) [a]		
		South Africa	Zambia	Uganda	Kenya	Nigeria	Zimbabwe	Thailand	Hong Kong	Malaysia
n (%)	2523 (100.0)	624 (24.7)	583 (23.1)	410 (16.3)	140 (5.5)	21(0.8)	17 (0.7)	542 (21.5)	111 (4.4)	75 (3.0)
A	340 (13.6)	2 (0.3)	4 (0.7)	235 (57.3)	97 (69.1)	1 (4.8)		1 (0.2)		
B	114 (4.5)	3 (0.5)						37 (7.0)	55 (49.5)	19 (25.3)
C	1236 (49.2)	617 (98.9)	571 (97.9)	9 (2.2)	19 (13.6)		17 (100.0)		2 (1.8)	1 (1.3)
D	179 (7.1)	1 (0.2)	1 (0.2)	160 (39.0)	17 (12.1)					
G	11 (0.4)		2 (0.3)	1 (0.2)	2 (1.4)	6 (28.6)				
CRF01_AE	585 (23.2)							485 (89.5)	52 (46.8)	48 (64.0)
CRF02_AG	20 (0.8)	1 (0.2)	3 (0.5)	1 (0.2)		14 (66.7)		1 (0.2)		
Other recombinants or discordant subtypes	32 (1.3)		2 (0.3)	1 (0.2)	2 (1.4)			18 (3.3)	2 (1.8)	7 (9.3)
Unclassified	6 (0.2)			3 (0.7)	3 (2.1)					

Data are n (%) of subtypes/CRFs, unless otherwise indicated. HIV subtypes were determined from the *pol* sequences, using the REGA HIV-1 subtyping algorithm version 2.0 (http://dbpartners.stanford.edu/RegaSubtyping) and/or Stanford HIV drug resistance database (http://hivdb.stanford.edu). CRF, circulating recombinant form. [a] The collaborating sites in Philippines, South Korea and Indonesia had not yet provided sequence data at time of the current analysis.

ponent, NVP was more commonly prescribed than EFV at 56% and 42%, respectively. First-line dual NRTI backbones were predominantly 3TC/d4T (47%), 3TC/AZT (29%) and 3TC/TDF (10%). For first-line PI regimens, the favored NRTI backbone was TDF/FTC (33%) compared to 3TC/d4T (30%) or 3TC/AZT (18%). For the ritonavir-boosted PI component, atazanavir (ATZ) was only slightly favored over LPV at 43% vs 41%, respectively. Overall, 542 (91.4%) of ARV naive patients started a 3TC-containing first-line regimen. The 10 PMTCT patients received perinatal prophylaxis of AZT/3TC/NVP (n=7), AZT (n=2) or AZT/NVP (n=1) for between 14 and 102 days and all were prescribed AZT/3TC/NVP as first-line regimens.

For the 25 (3.4%) second-line patients, 22 (88%) were of Thai ethnicity and the median age was 36.5 years (IQR 32.4 - 41.9). Females were in the majority (64%), HIV exposure was predominantly heterosexual (92%) and 60% of patients had experienced at least one CDC class C event. Of 21 patients with CD4 counts available within six months of starting a second-line therapy, the median CD4 count was 197 (IQR: 109-299). Median pre-switch HIV viral load was 4.0 $\log_{10}$ copies per/ml (IQR 3.6-4.5). All patients were on PI-based regimens, following failure on first-line NNRTI-based regimens (median duration 30.3 months). The most commonly prescribed PI was ritonavir-boosted LPV (88%).

As shown in table 5, from analysis of the 728 available viral sequences, the most common subtypes were CRF01_AE (584, 80.2%) and subtype B (111, 15.2%). Non-CRF01_AE recombinants were identified in 8 (1.1%) patient specimens. For 21 (2.9%) specimens, the subtype differed between PR and RT regions suggesting dual infection or recombination. International presentations have summarized 2009 baseline HIVDR mutations and subtype distributions [27, 28].

Complete baseline and prospective outcome data for PASER-M and TASER-M are anticipated to become available in 2010-2013.

WHAT ARE THE MAIN STRENGTHS AND WEAKNESSES?

Programmes that monitor national and regional levels of primary and secondary HIVDR contribute to evidence-based recommendations to inform treatment guidelines and provide feedback on the success of HIV treatment and prevention programmes. PASER and TASER, with TAQAS, are developing capacity in sub-Saharan Africa and the Asia-Pacific for coordinated HIVDR monitoring and genotypic laboratory testing. The study protocols are harmonized with the WHO HIV Drug Resistance Prevention Survey protocol [29]. An important strength is the large number of patients and sites participating, rep-

resenting a diverse spectrum of patient populations, clinic types, ART regimens and HIV subtypes. Opportunities exist to investigate the impact of drug resistance on HIV natural history, rates of disease progression and response to treatment in non-B subtypes. Data from genotypic resistance testing will also provide insight into the population genetics and dynamics of transmitted HIVDR in the region.

PASER-M and TASER-M have several limitations. First, patient samples at each site are not necessarily representative of the site, country or region. Second, data quality depends on the completeness of clinical information captured through routine patient care. In PASER-M, data may have been collected under varying conditions, since some sites had no or limited research experience at study initiation. Third, at some sites, study initiation was delayed by several months due to the time required for contract negotiation, IRB study approval and, in TASER-M, procurement of FWAs. After study initiation, recruiting the required number of patients within the recommended 18-month period proved difficult for some sites, due to asymptomatic patients not seeking care or treatment, cost of medication or low-prevalence in their setting. Fourth, HIVDR monitoring activities in resource-limited countries in sub-Saharan Africa are limited by high costs of laboratory testing. To address this challenge, PASER has initiated a public-private consortium, called Affordable Resistance Test for Africa (ART-A), which aims to evaluate affordable test algorithms for the detection and interpretation of HIVDR for use in laboratories and clinics (http://www.arta-africa.org).

HOW CAN I COLLABORATE? WHERE CAN I FIND OUT MORE?

Ownership of individual site data remains with the contributing site. Sites are represented by their principal investigators on the respective PASER and TASER Steering Committees. Research is to be the subject of peer-reviewed publications and analysis priorities are driven by a concept sheet process. Both studies accept concept proposals from external researchers for review, if submitted in collaboration with one or more of the site principal investigators. The PASER and TASER protocols contribute data under the LAASER partnership (http://www.laaser-hivaids.org) and TASER also contributes data to IeDEA [20]. Collaborating sites are also encouraged to make an appropriate subset of their data available to Ministry of Health in their respective country in order to contribute to local efforts in monitoring HIVDR. Questions regarding participation, research concepts or requests for data should be sent to Tobias Rinke de Wit, email: t.rinkedewit@pharmaccess.org (PASER), or Thida Singtoroj, email: thida.singtoroj@treatasia.org (TASER).

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APPENDIX

The PharmAccess African Studies to Evaluate Resistance

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AFRIKA

Part I

Transmitted HIV-1 drug resistance


AFRIKA

Chapter 5

HIV-1 drug resistance mutations are present in six percent of persons initiating antiretroviral therapy in Lusaka, Zambia

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ABSTRACT

Objective

To assess the mutational patterns and factors associated with baseline drug-resistant HIV-1 present at initiation of first-line antiretroviral therapy (ART) at 3 sites in Lusaka, Zambia, in 2007–2008.

Methods

Population sequencing of the HIV-1 pol gene was performed in the PharmAccess African Studies to Evaluate Resistance Monitoring cohort. Drug resistance–associated mutations (DRMs) were identified using the WHO 2009 Surveillance DRM list. Multiple logistic regression was used to assess factors associated with baseline resistance.

Results

The overall prevalence of baseline resistance was 5.7% [31 of 548 participants; 95% confidence interval (CI): 3.9 to 7.9]; the prevalence of DRMs associated with nucleoside reverse transcriptase inhibitors (NRTIs), non-nucleoside reverse transcriptase inhibitors (NNRTIs) and protease inhibitors was 1.1%, 4.0%, and 1.1%, respectively. Resistance prevalence was 5.2% (27 of 523) in antiretroviral-naive and 16.0% (4 of 25) in antiretroviral-experienced (ie, previous use of ART or antiretroviral prophylaxis for prevention of mother-to-child transmission) participants (P = 0.022). Dual-class resistance to NRTIs and NNRTIs was observed in 0.6% of participants. HIV-1 subtype C was identified in 98.0% (537 of 548) of participants. Prior antiretroviral experience (odds ratio: 4.32, CI: 1.34 to 14.0, P =0.015) and hemoglobin level (highest tertile versus lowest tertile odds ratio: 2.74, CI: 1.09 to 6.89, P = 0.033) were independently associated with baseline resistance.

Conclusions

Baseline resistance may compromise the response to standard NNRTI-based first-line ART in 6% of patients in Lusaka, Zambia. Continuous resistance monitoring is warranted to maintain individual and population-level ART effectiveness.

INTRODUCTION

Access to combination antiretroviral therapy (ART) for HIV-1–infected persons and antiretroviral (ARV) prophylaxis for prevention of mother-to-child transmission of HIV-1 (PMTCT) in sub-Saharan Africa has greatly expanded during the past 5 years [1]. In resource-limited settings where access to routine HIV-1 viral load monitoring is lacking and where the unregulated use of ARV drugs may be common, the selection of drug-resistant HIV-1 variants [2] and their subsequent transmission to newly infected individuals [3–6] is of particular concern, especially since second-line treatment options are limited. Few studies have assessed the mutational patterns associated with HIV-1 drug resistance among pre-treatment populations in sub-Saharan Africa in which drug pressure is increasing after ARV rollout [7–12].

The government of Zambia, a country in southern Africa, which is among the countries worst affected by the HIV-1 pandemic, initiated a comprehensive HIV-1 care and treatment program with support from international agencies [13]. By the end of 2007, nation-wide ART coverage was 46% of those in need [1]. Standard first-line ART regimens combine a dual nucleoside/nucleotide reverse transcriptase inhibitor (NRTI) backbone with a non-nucleoside reverse transcriptase inhibitor (NNRTI) [14].

The objective of this study was to evaluate the mutational patterns and factors associated with baseline drug resistance in HIV-1–infected individuals present at time of initiating first-line ART in the geographical setting of Lusaka, Zambia, where ART first became available in the country.

METHODS

Study population and design

The PharmAccess African Studies to Evaluate Resistance Monitoring Study is a multicenter prospective observational cohort of HIV-1–infected patients who receive ART in routine circumstances at 13 clinical sites in 6 African countries [15]. We conducted a cross-sectional analysis including 3 clinical sites in Lusaka, Zambia: Lusaka Trust Hospital, a private general hospital (Woodlands area); KARA Clinic, a free nongovernment sector clinic (city center); and, Coptic Hospital, a free faith-based general hospital (Manda Hill area). The 3 sites have provided HIV-1 care and treatment since 1997, 2004, and 2006, respectively. The Academic Medical Center Institutional Review Board and the University of Zambia Research Ethics Committee approved all study procedures. Confirmed HIV-1 seropositive individuals aged ≥18 years who were eligible to initiate first-line ART

as defined by national guidelines (i.e., advanced immunodeficiency as defined by CD4 count, 200 cells/mL or advanced disease according to the World Health Organization (WHO) clinical stages) [14] were consecutively enrolled. All participants provided written informed consent for use of routinely collected demographic, clinical, and laboratory data and additional phlebotomy for assessment of HIV-1 RNA and genotypic resistance. Exclusion criteria were pregnancy at study screening and re-initiation on first-line ART less than 30 days after stopping previous first-line ART. Re-initiation on first-line ART more than 30 days after stopping previous first-line ART and/or any previous use of ARV prophylaxis or non-suppressive mono/dual therapy were not exclusion criteria (ARV-experienced group).

Laboratory methods

HIV-1 RNA determination was performed on EDTA-anticoagulated plasma using the NucliSens EasyQ real-time assay version 1.2 (bioMérieux, Lyon, France). Population-based sequencing was performed on all plasma specimens which had HIV-1 RNA >1000 copies per milliliter using an in-house method [16]. Briefly, HIV-1 RNA was extracted from 200 mL of plasma using the automated Roche MagNa Pure LC analyzer and the MagNA Pure LC Total Nucleic Acid Isolation Kit (Roche, Germany). Genotyping encompassed protease and codons 1–230 of reverse-transcriptase, using an in-house sequencing method with an ABI 3730 Genetic Analyzer (Applied Biosystems, Foster City, CA). Sequences were assembled and manually edited using Sequencher version 4.5 software (Genecodes, Ann Arbor, MI). GenBank accession numbers: HM119603–HM120150.

Genotypic resistance analysis and subtyping

Baseline resistance was defined as the presence of ≥1 Drug resistance–associated mutation (DRM) according to the WHO 2009 Surveillance Drug Resistance Mutation list [17] using the Stanford Calibrated Population Resistance analysis tool (version 4.1 beta, available at http://hivdb.stanford.edu/). HIV-1 subtypes were determined using the REGA HIV-1 subtyping algorithm (version 2.0, available at http://www.bioafrica.net/subtypetool/html/) [18] and additional phylogenetic analysis using neighbor-joining method if required.

Statistical methods

Univariate and multivariate analyses were used to determine factors associated with drug resistance using logistic regression and expressed as odds ratios (ORs) (95% confidence interval, CI) and P values ($P < 0.05$ statistically significant). Prevalence values were calculated with a CI based on the binomial distribution. Categorical data were compared using Chi^2 test. Continuous data were investigated using Student t test. All analyses were performed using Stata version 10 (StataCorp LP, TX).

RESULTS

Study population

Between March 2007 and September 2008, a total of 839 adult men and non-pregnant women were recorded to initiate first-line ART at the 3 sites. Screening efforts resulted in the enrolment in the study of 584 individuals who met eligibility criteria and provided consent (i.e., 70% recruitment rate). A valid baseline HIV-1 RNA result was available for 576 (98.6%) participants. HIV-1 RNA was >1000 copies per milliliter in 556 (96.5%); of those, sequence analysis was successful in 548 (98.6%). Table 1 summarizes the demographic characteristics of all 584 participants. Females comprised 54.8% (n = 320) and were younger than males [36.1 (SD 8.8) versus 40.0 (SD 8.8) years, P<0.0001]. All participants were native Zambians. 321 (55.0%) participants had advanced stage disease (ie, WHO stage III or IV). Median CD4 count was 132 cells per microliter (interquartile range, IQR: 69–203). Mean HIV-1 RNA was 5.0 (SD: 0.9) $\log_{10}$ copies/mL. Twenty-seven (4.6%) participants had previous ARV-experience, either as (highly active) ART (n = 14), single-dose nevirapine for PMTCT (n = 5), combination therapy for PMTCT (n = 1), or unspecified (n = 7). Patient characteristics neither differed between ARV-naive versus ARV-experienced participants (table 1) nor between sites (data not shown).

Table 1. Characteristics of participants (n=584) with and without previous antiretroviral experience *

Characteristic	Total	ARV naive	ARV experienced *	p-value
Participants – no. (%)	584	557 (95.4)	27 (4.6)	
Age (years) – mean (sd)	37.9 (9.0)	38.0 (9.1)	35.4 (7.6)	.1454
Sex – no. (%)				
Female	320 (54.8)	301 (54.0)	19 (70.4)	.096
Male	264 (45.2)	256 (46.0)	8 (29.6)	
WHO clinical stage – no. (%)				.318
Early (I/II)	263 (45.0)	252 (45.2)	11 (40.7)	
Advanced (III/IV)	321 (55.0)	305 (55.8)	16 (59.4)	
Hemoglobin (g/dL) - median (IQR) †	11.2 (9.9-12.7)	11.2 (2.7)	11 (2.9)	.6645
CD4 count (cells/µl) - median (IQR) ᶜ	132 (69-203)	130 (63-193)	152 (81-223)	.4063
HIV-1 RNA ($\log_{10}$ c/mL) - mean (SD) ‡	5.0 (.9)	4.9 (.9)	5.1 (.7)	.2993

Data are no. (%) of participants, unless otherwise indicated. *Previous antiretroviral experience was defined as re-initiation on first-line ART (more than 30 days after stopping previous first-line ART), and/or any previous use of ARV prophylaxis or non-suppressive mono/dual therapy; previous ARV experience among n = 27 participants comprised previous (highly active) ART (n = 14), single-dose nevirapine for PMTCT (n = 5), combination therapy for PMTCT (n = 1), and unspecified (n = 7). †Data available for n = 580. ‡Data available for n = 576. ART, antiretroviral therapy; ARV, antiretroviral; IQR, interquartile range; NVP, nevirapine; WHO, World Health Organization.

Frequencies of subtypes and drug resistance–associated mutations

Subtype C was identified in 98.0% of participants (537 of 548). Other subtypes and circulating recombinant forms (CRFs) were subtype A1 (0.5%, 3 of 548), CRF02_AG (0.5%, 3 of 548), G (0.4%, 2 of 548), CRF09_cpx (0.4%, 2 of 548), and D (0.2%, 1 of 548). The overall prevalence of resistance was 5.7% (31 of 548 participants; CI: 3.9% to 7.9%); the prevalence of DRMs associated with NRTIs, NNRTIs, and protease inhibitors (PIs) was 1.1% (6 of 548), 4.0% (22 of 548), and 1.1% (6 of 548), respectively (figure 1). Among ARV-naive participants, the prevalence of resistance was 5.2% (27 of 523; CI: 3.4% to 7.4%); the prevalence of DRMs associated with NRTIs, NNRTIs, and PIs was 1.0% (5 of 523), 3.6% (19 of 523), and 1.1% (6 of 523), respectively. Among ARV-experienced participants, the prevalence of resistance was 16.0% (4 of 25 participants; CI: 4.5% to 36.1%); the prevalence of DRMs associated with NRTIs, NNRTIs, and PIs was 4.0% (1 of 25), 12.0% (3 of 25), and 0.0% (0 of 25), respectively. Detected DRMs were Y181C (1.6%, 9 of 548), K103N (1.3%, 7 of 548), K103S (0.7%, 4 of 548), G190A (0.7%, 4 of 548), L100I (0.4%, 2 of 548), K101E (0.4%, 2 of 548), M184V (0.4%, 2 of 548), V106M (0.2%, 1 of 548), Y188C (0.2%, 1 of 548), G190S (0.2%, 1 of 548), K65R (0.2%, 1 of 548), T69D (0.2%, 1 of 548), K70R (0.2%, 1 of 548), K70E (0.2%, 1 of 548), L74I (0.2%, 1 of 548), V75S (0.2%, 1 of 548), V75T (0.2%, 1 of 548), and K219E (0.2%, 1 of 548) in reverse transcriptase and L90M (0.7%, 4 of 548), I85V (0.2%, 1 of 548), and I50L (0.2%, 1 of 548) in protease. DRM frequencies did not differ significantly across sites (data not shown). Table 2 provides an overview of demographic and virologic characteristics of the 31 participants who harbored ≥1 DRM. Dual-class re-

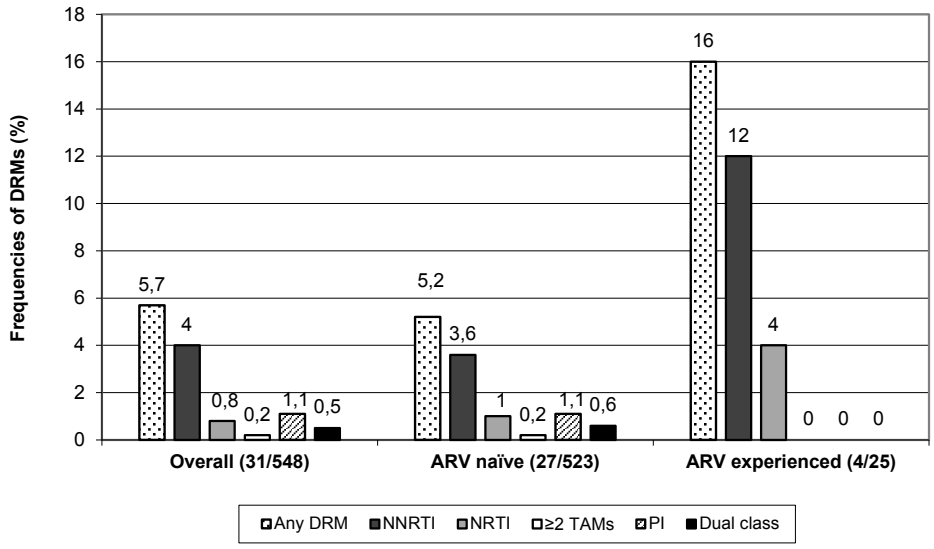

Figure 1. Frequencies of drug resistance–associated mutations in all participants and separately for antiretroviral-naive and antiretroviral-experienced participants.

Table 2. Demographic and virologic characteristics of participants (n=31) who harbored ≥1 drug resistance-associated mutation.

#	Specimen ID	Age (years)	Sex	ARV history	CD4 count (cells/µl)	HIV-1 RNA (log_{10} c/ml)	Genetic sub-type*	Drug resistance-associated mutations†		
								NRTI	NNRTI	PI
1	00011	27	female	naive	42	5.5	C		Y181C, G190A	
2	00035	25	female	naive	122	3.8	C		L100I	
3	00061	48	male	naive	98	4.8	C	M184V	K103S, V106M	
4	00067	38	female	naive	32	5.9	C		K103N, Y181C	
5	00068	40	male	naive	193	5.0	C		G190S	
6	00073	49	male	naive	156	5.3	C			L90M
7	00083	40	female	naive	46	5.9	C			L90M
8	00138	29	female	naive	83	5.7	C		K101E	
9	00143	32	female	single-dose NVP (2002)	169	5.2	C	L74I, V75S		
10	00145	37	female	3TC+d4T+NVP (Sep05-Jun06)	9	5.9	C		G190A	
11	00182	39	female	naive	99	5.7	C		K103N	
12	00197	40	male	naive	16	4.7	C			I50L
13	00279	38	female	naive	26	4.5	C	K65R, V75T	Y181C	
14	00409	18	male	naive	187	5.0	C			L90M
15	00417	36	male	naive	219	4.3	C			L90M
16	00447	36	female	naive	211	4.2	C		L100I	
17	00455	38	female	naive	161	5.2	C		G190A	
18	00483	22	female	naive	122	5.3	C		K103N	
19	00515	56	male	naive	24	3.6	C		K103N, K103S	
20	00521	55	female	naive	159	4.1	C		K103S	
21	00539	42	male	naive	77	4.1	C		Y181C	

Table 2 (continued)

#	Specimen ID	Age (years)	Sex	ARV history	CD4 count (cells/µl)	HIV-1 RNA (log_{10} c/ml)	Genetic subtype *	Drug resistance-associated mutations†		
22	00570	34	male	naive	118	4.9	C	T69D		
23	00580	35	male	AZT+3TC+NVP (Mar-May06)	159	5.0	C		Y181C	
24	00624	37	male	naive	249	4.0	C		K103N	
25	00652	27	female	naive	106	4.7	C	K70R, M184V, K219E	K101E, Y181C, G190A	
26	00666	30	male	AZT+3TC+NVP (Nov05-Apr06)	216	4.4	C		Y181C	
27	00670	45	male	naive	44	5.3	C			I85V
28	00671	32	female	naive	181	4.3	C		K103S	
29	02368	33	female	naive	93	6.2	C		K103N, Y181C	
30	02449	39	male	naive	80	5.1	C		K103N, Y181C, Y188C	
31	03977	46	male	naive	81	4.9	C	K70E		

* HIV-1 subtypes were determined from the pol sequences using the REGA HIV-1 subtyping algorithm (version 2.0, available at http://www.bioafrica.net/subtypetool/html/) and, for sequences with ambiguous subtype assignment, additional phylogenetic analysis (neighbor-joining method). † HIV-1 genotypic sequence analysis encompassing protease and partial reverse transcriptase; drug-resistance–associated mutations according to the WHO 2009 Surveillance Drug Resistance Mutation list. AZT, zidovudine; 3TC, lamivudine; d4T, stavudine; NVP, nevirapine

Table 3. Factors associated with HIV-1 genotypic baseline drug resistance.

				Univariate		Multivariate	
Characteristic *	**Total**	**DR**	**No DR**	**OR (95% CI)**	**p**	**OR (95% CI)**	**p**
Participants – no. (%)	548	31 (5.7)	517 (94.3)				
Age (years) – mean (SD)	37.8 (8.9)	37.3 (8.8)	37.8 (8.9)	.99 (.95 to 1.03)	.732		
Sex – no. (%)							
Female	296 (54.0)	16 (51.6)	280 (54.2)	reference			
Male	252 (46.0)	15 (48.4)	237 (45.8)	1.11 (.54 to 2.3)	.782		
WHO clinical stage III/IV – no. (%)							
Early (I/II)	244 (44.5)	17 (54.8)	227 (43.9)	reference			
Advanced (III/IV)	304 (55.5)	14 (45.2)	290 (56.1)	.64 (.31 to 1.34)	.237		
History of ARV drug use – no. (%)							
ARV naive	523 (95.4)	27 (87.1)	496 (95.9)	reference		reference	
ARV experienced †	25 (4.6)	4 (12.9) [a]	21 (4.1)	3.50 (1.12 to 10.9)	.031	4.32 (1.34 to 14.0)	.015 ‡
Site							
LTH	109 (19.9)	6 (19.4)	103 (19.9)	reference			
KC	213 (38.9)	13 (41.9)	200 (38.7)	1.12 (.41-3.02)	.829		
CH	226 (41.2)	12 (38.7)	214 (41.4)	.96 (.35-2.64)	.941		
HIV-1 subtype					1.0 §		
C	537 (98.0)	31 (100.0)	506 (97.9)	reference			
Other «	11 (2.0)	0 (0.0)	11 (2.1)	n/a [f]	n/a §		
Hemoglobin (g/dL) - median (IQR) ¶	11.1 (9.8-12.7)	11.9 (10.5-13.4)	11.1 (9.6-12.6)				
Lowest tertile	189 (34.7)	8 (25.8)	181 (35.3)	reference		reference	
Middle tertile	178 (32.7)	8 (25.8)	170 (33.1)	1.06 (.39 to 2.90)	.902	1.20 (.43 to 3.32)	.723
Highest tertile	177 (32.5)	15 (48.4)	162 (31.6)	2.09 (.87 to 5.07)	.101	2.74 (1.09 to 6.89)	.033 ‡
CD4 count (cells/µl) - median (IQR)	129.5 (68 – 200)	106 (44.5-167.5)	132 (65-199)	.73 (.47 to 1.13) #	.162	0.65 (.41 to 1.02) #	.063
HIV-1 RNA (log_{10} c/mL) - mean (SD)	5.06 (0.7)	4.91 (0.7)	5.07 (0.8)	.76 (.48 to 1.22)	.261		

Data are no. (%) of participants, unless otherwise indicated. *Characteristics describe participants from whom a baseline HIV-1 genotypic sequence analysis was available (n = 548). † Previous antiretroviral experience was defined as re-initiation on first-line ART more than 30 days after stopping previous first-line ART and/or any previous use of ARV prophylaxis or non-suppressive mono/dual therapy; ARV-experience among n = 25 participants with versus without DR comprised previous (highly active) ART (n = 3 versus 10), single-dose nevirapine for PMTCT (n = 1 versus 4), combination therapy for PMTCT (n = 0 versus 1) and unspecified (n = 0 versus 6). ‡Statistically significant results (P<0.05).§ Logistic analysis not valid for this variable; P value by Fisher exact. « Other subtypes and circulating recombinant forms comprised A1 (n = 3), CRF02_AG (n = 3), G (n = 2), CRF09_cpx (n = 2), and D (n = 1). ¶ Data available for n = 544. # OR for a 100-cell increase of CD4 count. ART, antiretroviral therapy; ARV, antiretroviral; CH, Coptic Hospital; CI, confidence interval; CRFs, circulating recombinant forms; DR, genotypic drug resistance; IQR, interquartile range; KC, KARA Clinic; LTH, Lusaka Trust Hospital; n/a, not applicable; NVP, nevirapine; OR, odds ratio; WHO, World Health Organization.

sistance to NRTIs and NNRTIs was detected in 0.6% (3 of 523) of ARV-naive participants, and no triple-class resistance was observed. One ARV-naive participant harbored M184V plus 2 thymidine analogue mutations (TAMs) by the TAMII pathway (K70R and K219E).

Factors associated with baseline drug resistance

In univariate analysis, participants with versus without resistance did not differ for age, sex, WHO clinical stage, median serum hemoglobin level, median CD4 count, mean HIV-1 RNA, and subtype, whereas previous ARV experience was more frequent among participants with drug resistance compared with those without resistance (12.9% versus 4.1%, P = 0.031) (table 3). Multiple logistic regression analysis of patient characteristics demonstrated that previous ARV use (versus ARV-naive status; OR: 4.32, CI: 1.34 to 14.0, P = 0.015) and hemoglobin level (highest tertile versus lowest tertile; OR: 2.74, CI: 1.09 to 6.89, P = 0.033) were independently associated with the presence of baseline resistance (table 3).

DISCUSSION

In this study, we investigated patterns of drug-resistant HIV-1 present at time of initiating first-line ART among 584 predominantly HIV-1 subtype C–infected patients in Lusaka, Zambia, enrolled in the PharmAccess African Studies to Evaluate Resistance Monitoring study during 2007–2008. Six (CI 3.9 to 7.9) percent of participants were found to harbor ≥1 DRM, based on population genotyping. Baseline resistance may reflect the combined effect of drug-resistant strains transmitted during infection and acquired during previous ARV exposure. The majority (95.4%) of participants were reported to be ARV naive at baseline; compared with ARV-naive status, resistance was more frequent after prior use of ART or PMTCT (16.0% versus 5.2%, P = 0.022). NNRTI-associated DRMs were observed in the highest frequency (4.0%), whereas dual-class or triple-class resistance was rarely observed. An important strength of the study was its large multisite patient sample, representative of a variety of clinic populations within the geographic setting of Lusaka.

Primary HIV-1 drug resistance in Zambia had only been assessed in 1 small study before ARV drugs became widely available, reporting no major drug resistance–associated mutations (DRMs) among 28 ARV-naive persons [19]. Population surveys [7–12] and mathematical models [20] to date have reported low levels of drug-resistant infections in African populations with increasing selective ARV drug pressure, but recent preliminary reports have suggested transmission of resistant strains directly after national ARV rollout programs [21, 22]. The predominance of NNRTI-associated DRMs (mostly Y181C and K103N/S) in this baseline study probably results from the widespread use of NNRTIs

as part of standard first-line ART and PMTCT regimens. Most solitary NNRTI-associated DRMs cause a complete loss of activity of efavirenz and nevirapine [23] which may compromise the initial response to the standard first-line therapy [22]. Moreover, NNRTI-associated DRMs have been shown to have only a modest impact on viral replicative fitness [23, 24], allowing them to persist in the absence of the drug and to establish infection in a new host. Sex was not found to be associated with NNRTI resistance, suggesting either a limited effect on baseline resistance from PMTCT in females or secondary transmission of PMTCT-acquired NNRTI-resistant strains.

We found baseline NRTI-associated resistance to be limited. This contrasts not only with industrialized countries where transmission of NRTI-associated resistance is predominant [3–6] but also with several recent African studies in subtype C–infected patients experiencing treatment failure which reported considerable rates of NRTI-associated DRMs, including TAMs and K65R [25–28]. Several mechanisms could be expected to play a role. First, ARV rollout in sub-Saharan Africa is based on (highly active) ART, and widespread access has been established only recently, whereas in the industrialized world, ARV drugs have been widely used for many years including non-suppressive mono and dual therapies with thymidine analogues in the past. Second, the reduced replicative fitness of variants harboring multiple TAMs and/or K65R might reduce transmission efficiency [29]. Third, underestimation of NRTI-associated DRMs is possible due to reversion to wild type and/or outgrowth of minority wild-type species over time in absence of drug-selective pressure resulting in a reduction of the mutant strains with poor replicative fitness to minor variants below the limit of detection of genotypic analysis [30–33].

Because of the infrequent use of PIs, we observed very few significant DRMs in protease. A few participants harbored clades D, G, CRF02_AG, and CRF09_cpx, which to our knowledge have not been described in Zambia before. Twenty specimens (3.5%) had unexpectedly low (<1000 copies/mL) HIV-1 RNA levels, which is most likely due to inter-individual variations in viral replication rates and immunologic control [34]; other reasons could include partial viral suppression because of any undisclosed recent ARV exposure and varied assay performance between viral subtypes [35–37]. As an additional observation, the presence of drug-resistant virus at baseline was found to be associated with a non-reduced serum hemoglobin level. A non-causal relation seems plausible, that is, advancing HIV infection leading to anemia as a result of bone marrow suppression [38], in parallel to the diminution of poorly replicating drug-resistant minor variants, as described above [30–33].

The study has several limitations. First, it cannot be completely ruled out that reportedly ARV-naive participants had unknown previous exposure to therapy and/or prophylaxis.

Second, the potential for selection bias exists, although the lack of heterogeneity in resistance pattern between the established private versus the 2 more recently introduced free ART programs argues against this. Third, data on route, country and duration of HIV-1 infection, and the source's ARV history, as possible associated factors of resistance, were not available.

In conclusion, this study on baseline HIV-1 resistance in routine ART programs in Lusaka, Zambia, adds important information regarding the predicted population-level response to standard first-line ART. Patients with previous ARV-experience are particularly at risk for a compromised initial response to standard NNRTI-based regimens. If baseline NNRTI resistance levels further increase, reassessment of first-line guidelines may be warranted to maintain individual and population-level benefits of ART. It is mandatory to monitor worldwide for the presence and spread of drug-resistant HIV-1.

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Contributors

R.L.H., M.S., C.L.W., W.S.S., M.V.V., R.S., T.F.R.D.W. designed the study. M.S., M.L., R.V.H. acquired the data. C.L.W., W.S.S. performed the laboratory testing. R.L.H., T.F.R.D.W. analyzed and interpreted the data. R.L.H. wrote the first draft of the article. C.L.W., R.S., A.M.J.W., T.F.R.D.W. critically revised the article for important intellectual content. All authors contributed to subsequent drafts and reviewed and approved the final article.

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Chapter 6

HIV-1 drug resistance in antiretroviral-naive individuals in sub-Saharan Africa after rollout of antiretroviral therapy: a multicentre observational study

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ABSTRACT

Background

There are few data on the epidemiology of primary HIV-1 drug resistance after the roll-out of antiretroviral treatment (ART) in sub-Saharan Africa. We aimed to assess the prevalence of primary resistance in six African countries after ART roll-out and if wider use of ART in sub-Saharan Africa is associated with rising prevalence of drug resistance.

Methods

We did a cross-sectional study in antiretroviral-naive adults infected with HIV-1 who had not started first-line ART, recruited between 2007 and 2009 from 11 regions in Kenya, Nigeria, South Africa, Uganda, Zambia, and Zimbabwe. We did population-based sequencing of the *pol* gene on plasma specimens with greater than 1000 copies per mL of HIV RNA. We identified drug-resistance mutations with the WHO list for transmitted resistance. The prevalence of sequences containing at least one drug-resistance mutation was calculated accounting for the sampling weights of the sites. We assessed the risk factors of resistance with multilevel logistic regression with random coefficients.

Findings

2436 (94.1%) of 2590 participants had a pretreatment genotypic resistance result. 1486 participants (57.4%) were women, 1575 (60.8%) had WHO clinical stage 3 or 4 disease, and the median CD4 count was 133 cells per µL (IQR 62–204). Overall sample-weighted drug-resistance prevalence was 5.6% (139 of 2436; 95% CI 4.6–6.7), ranging from 1.1% (two of 176; 0.0–2.7) in Pretoria, South Africa, to 12.3% (22 of 179; 7.5–17.1) in Kampala, Uganda. The pooled prevalence for all three Ugandan sites was 11.6% (66 of 570; 8.9–14.2), compared with 3.5% (73 of 1866; 2.5–4.5) for all other sites. Drug class-specific resistance prevalence was 2.5% (54 of 2436; 1.8–3.2) for nucleoside reverse-transcriptase inhibitors (NRTIs), 3.3% (83 of 2436; 2.5–4.2) for non-NRTIs (NNRTIs), 1.3% (31 of 2436; 0.8–1.8) for protease inhibitors, and 1.2% (25 of 2436; 0.7–1.7) for dual-class resistance to NRTIs and NNRTIs. The most common drug-resistance mutations were K103N (43 [1.8%] of 2436), thymidine analogue mutations (33 [1.6%] of 2436), M184V (25 [1.2%] of 2436), and Y181C/I (19 [0.7%] of 2436). The odds ratio for drug resistance associated with each additional year since the start of the ART roll-out in a region was 1.38 (95% CI 1.13–1.68; p=0.001).

Interpretation

The higher prevalence of primary drug resistance in Uganda than in other African countries is probably related to the earlier start of ART roll-out in Uganda. Resistance surveillance and prevention should be prioritised in settings where ART programmes are scaled up.

Funding

Ministry of Foreign Affairs of the Netherlands.

INTRODUCTION

Sub-Saharan Africa has the highest prevalence of HIV-1 worldwide and access to combination antiretroviral treatment (ART) has expanded in recent years to reach millions of infected people, although access is not universal [1]. The use of standardised and affordable first-line combinations of antiretroviral drugs through a public health approach, including two nucleoside reverse transcriptase inhibitors (NRTIs) and one non-nucleoside reverse transcriptase inhibitor (NNRTI), has been crucial to allow the scale-up of ART [2]. However, concern has been raised about the public health implications of the emergence of resistance to antiretroviral drugs [3]. Mutations in the HIV genome that confer drug resistance, acquired during ART failure, might limit the response to subsequent lines of treatment. The threat of increased onward transmission of drug-resistant strains to newly infected people—primary drug resistance—has the potential to compromise the effectiveness of first-line ART regimens [4-7].

In developed countries, the wider use of ART has been associated with an initial increase [5,7,8] and subsequent stabilisation [9,10] of levels of primary resistance to NRTIs and NNRTIs. In Europe [9] and the USA [10] an estimated 9–15% of antiretroviral-naive people harbour viruses with at least one drug-resistance mutation, and therefore pretreatment resistance testing is recommended to guide individual therapy choices [11,12].

In developing countries, the use of less potent and less tolerable ART regimens, restricted access to virological monitoring, and inconsistent drug supply could accelerate the emergence of resistance [3,13]. Few data exist on the epidemiology of primary resistance after the scale-up of ART in sub-Saharan Africa, and the ability to compare previous studies has been limited by differences in study populations, time periods, and definitions of drug resistance. The prevalence of primary resistance has been estimated to be low (<5%) in surveys of individuals newly diagnosed with HIV in several African countries [14] and by mathematical modelling [15]. However, recent reports have suggested an increase in primary resistance in east and southern Africa, in parallel with the widespread distribution of ART [16,17]. Furthermore, the extent to which the genetic diversity in HIV-1 subtypes and recombinants in Africa might affect the emergence of resistance is controversial [18].

To establish the extent of HIV-1 drug resistance in sub-Saharan Africa, the PharmAccess African Studies to Evaluate Resistance Monitoring (PASER-M) cohort was started in 2007 [19]. The aim of the present study was to establish the prevalence, distribution, and risk factors of primary drug-resistance in antiretroviral-naive individuals infected with HIV-1 in six African countries. We specifically sought to assess if the wider use of ART in sub-Saharan Africa is associated with an increasing prevalence of drug resistance in pretreatment populations.

METHODS

Participants

PASER-M is a multicentre, prospective cohort of individuals infected with HIV-1 receiving first-line or second-line ART in routine circumstances at 13 clinical sites situated in 11 regions, mainly major cities or urban areas, in six African countries (three sites in South Africa, three in Uganda, three in Zambia, two in Kenya, one in Nigeria, and one in Zimbabwe). The median site-specific enrolment period was 12 months (range 6–18 months) between March, 2007, and September, 2009. Cohort and site characteristics have been profiled elsewhere [19]. Our study focused on the epidemiology of primary drug-resistance per region. For the three collaborating sites situated in Lusaka, Zambia, we previously reported similar primary resistance prevalence and patterns [20]; therefore, for the purpose of our analysis we deemed it appropriate to group them into one region.

For our cross-sectional baseline analysis, we included PASER-M study participants if they were aged 18 years or older, infected with HIV-1, and eligible to start first-line ART in accordance with national guidelines—ie, advanced immunodeficiency (CD4 cell count <200 cells per μL) or advanced HIV disease (WHO clinical stage 3 or 4) [21]. We excluded individuals who reported previous use of antiretroviral drugs for treatment or prophylaxis. We reassessed, with a standard questionnaire, the antiretroviral drug histories of all individuals who were identified as harbouring HIV-1 with at least one detectable drug-resistance mutation, to minimise possible bias from the misclassification of individuals with undisclosed previous exposure to antiretroviral drugs as drug-naive. Our other exclusion criteria were pregnancy at study screening, or, in Nigeria, HIV-2 co-infection.

Participants provided written informed consent at enrolment. Participants were sequentially enrolled during a site-specific enrolment period of a maximum of 18 months. The study protocol was approved by the appropriate national and local research ethics committees at the collaborating sites and the Academic Medical Centre of the University of Amsterdam, Netherlands.

Procedures

Medical staff at each site extracted routine clinical and laboratory data recorded in medical records into standard case-report forms, which were double-entered into a central web-based database. For recorded values of CD4 cell counts, the most recent measurement before the date of enrolment was defined as the pre-treatment count.

To assess the possible population-level effect of ART programmes on the prevalence of primary resistance, we assessed the time that elapsed since the initial roll-out for each region as a proxy measure for the amount of circulating drug-resistant HIV-1 variants in the general population. We calculated the ecological time variable for each participant as the number of years elapsed between start of ART rollout in each region (estimated as July 1 of the calendar year) and the date of sampling of patients. We obtained information on the calendar year of start of ART roll-out in each region from UN General Assembly special session country reports[22] and the respective site study teams (calendar year range 2000–04).

EDTA (edetic acid)-anticoagulated plasma specimens were collected before the start of ART, and stored for later assessment of HIV RNA viral-load and genotypic drug-resistance testing. We did population-based sequencing of HIV-1 protease and codons 1–300 of reverse transcriptase on all specimens in which viral load was greater than 1000 copies per mL. Virological testing was done at two reference laboratories in South Africa (serving the sites in Kenya, Nigeria, South Africa, Zambia, and Zimbabwe) and Uganda (serving the sites in Uganda). Viral-loads were established with NucliSens EasyQ real-time assay (version 2.0; bioMérieux, Lyon, France) in South Africa or COBAS Ampliprep/COBAS Taqman assay (Roche, Branchburg, NJ, USA) in Uganda. Genotyping was done with in-house sequencing methods with an ABI Prism 3730 Analyzer (Applied Biosystems, Foster City, CA, USA) in South Africa [23] or BC CEQ 8000 Analyzer (Beckman Coulter Inc, Fullerton, CA, USA) in Uganda [24]. Sequences were manually edited with Sequencher (version 4.8; Genecodes, Ann Arbor, MI, USA) in South Africa or BioEdit (version 7.0.9.0) in Uganda. Both laboratories participated in external quality assessment schemes for genotypic drug-resistance testing [25]. The quality of the sequences was verified with ViroScore Suite (version 8.4; ABL SA, France). Drug-resistance mutations were identified on the basis of the 2009 WHO list for surveillance of transmitted resistance [26], with the Stanford Calibrated Population Resistance Analysis Tool [27]. We judged that sequences with genetic mixtures of wild-type and mutant sequences at amino-acid sites that code for drug-resistance mutations contained a resistant strain. HIV-1 subtypes were inferred from the *pol* sequences with the REGA algorithm [28] and confirmed with the STAR algorithm [29]. To predict the effect of the identified drug-resistance mutations on drug susceptibility, we used the Stanford drug-susceptibility algorithm (version 6.0.9) [26] to

classify sequences as susceptible (Stanford level 1 or 2), low-level resistance (Stanford level 3), intermediate-level resistance (Stanford level 4), or high-level resistance (Stanford level 5) to the drug classes and specific drugs. All sequences have been deposited in GenBank (webappendix panel).

Statistical methods

Because our original study design was a prospective cohort, we estimated a sample size of at least 190 individuals per site on the basis of the predicted virological outcome after 24 months of ART, accounting for attrition [19]. Assuming 95% (n=181) of individuals to be antiretroviral-naive before the start of ART [20] and a prevalence of primary resistance of 5% [16] the statistical power was 87% to discriminate the prevalence of resistance to within 4% with a 95% CI of 2.6–9.5, with a two-sided significance level of 5%. We compared categorical data with the χ^2 test and continuous data with the Kruskal Wallis test or one-way ANOVA, where appropriate. We calculated the prevalence of sequences containing at least one drug-resistance mutation accounting for the sampling weights of the sites, and further specified for each drug class (ie, NRTI, NNRTI, and protease inhibitors). We expressed prevalence estimates with a 95% CI based on the normal approximation to the binomial distribution.

We used multilevel analysis with random coefficients to assess the effects of explanatory variables, at the levels of individuals and sites (while accounting for the possible interdependence of observations clustered within sites) on two outcomes: any resistance and NNRTI-resistance. We assessed all variables separately and entered those associated ($p<0.1$) with the outcomes stepwise into the multivariate model. We assessed biologically plausible interactions in the multivariate model. Co-variables investigated were age, sex, WHO clinical stage, CD4 cell count (fitted as a continuous variable), HIV RNA load ($\log_{10}$ transformed, fitted as a continuous variable), HIV-1 subtype, HIV exposure category, patients' performance status, clinical site administration, calendar year of sampling, and the time since start of ART roll-out in the region (fitted as a continuous variable). We expressed our results as odds ratios (ORs) with 95% CI and two-sided p values, with $p<0.05$ being statistically significant. We did all analyses with Stata version 10.

Role of the funding source

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

RESULTS

Viral load measurements before treatment were available for 2574 (99.0%) of 2601 participants included in our study, of whom 2478 (96.3%) had a value of greater than 1000 copies per mL (figure 1). Upon reassessment of the antiretroviral drug histories of the 150 participants (6.1%) who harboured virus with at least one drug-resistance mutation, 96 were confirmed antiretroviral-naive, 11 reported previous antiretroviral drug use (five ART, one post-exposure prophylaxis, and six prophylaxis for prevention of mother-child transmission), and 43 had an unconfirmed previous antiretroviral drug status because they had died or were lost to follow-up. After exclusion of all participants with previous

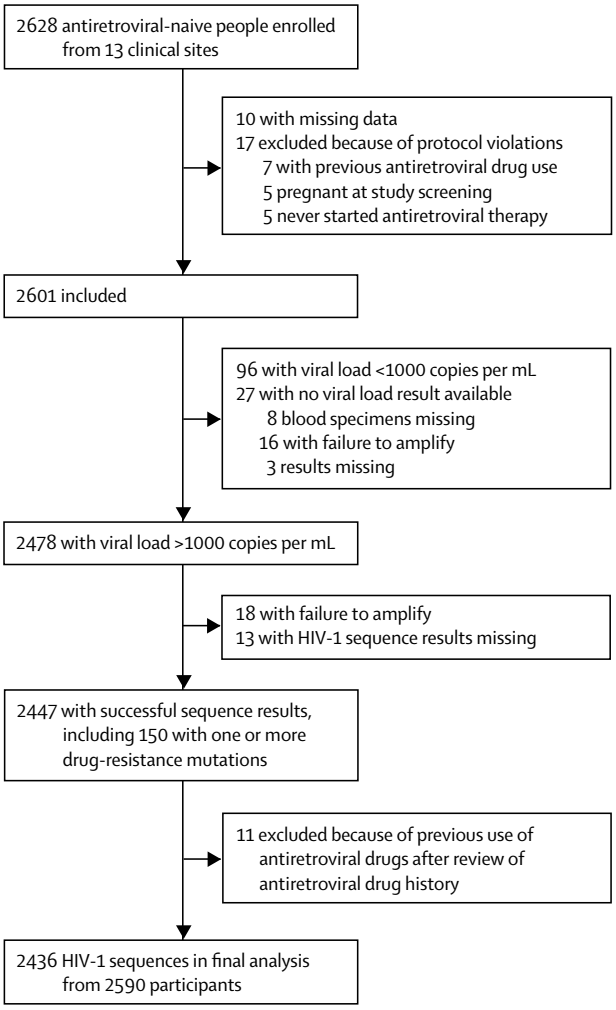

Figure 1. Study profile

antiretroviral exposure, the final analysis included 2590 participants, and a pretreatment sequence result was available for 2436 (94.1%; figure 1).

The median number of participants per site was 213 (range 182–552) and 57.4% were women (table 1). Mean age was 38.0 years (SD 9.0), and women were younger than men (36.2 years [SD 8.6] *vs* 40.3 years [SD 9.0]; p<0.0001). HIV acquisition was predominantly through heterosexual contact (66.5%) or unknown (32.8%). Nearly all participants (2570; 99.2%) were native residents in their countries. Advanced HIV disease (WHO stage 3 or 4) was present in 60.8% of participants (table 1). Initial first-line ART regimens were almost exclusively NNRTI-based (2582; 99.7%), with dual-NRTI-backbones consisting of zidovudine (962; 37.2%), tenofovir (866; 33.5%), stavudine (688; 26.6%), or abacavir (66; 2.6%) combined with either lamivudine (1740; 67.4%) or emtricitabine (842; 32.6%). The remaining patients were prescribed regimens based on protease inhibitors (six; 0.2%) or triple NRTIs (two; 0.1%). The median time elapsed since the start of ART roll-out in the region was 4.7 years (IQR 4.0–6.0; range 2.8–8.0).

HIV-1 subtype C was most commonly identified followed (in descending order) by A, D, A/G recombinant, G, and other subtypes and recombinants (table 1); we classed six as other subtypes (0.3%) and 44 as other recombinants (1.8%). Subtype C was predominant in the regions of South Africa (98.0%), Zambia (97.7%), and Zimbabwe (99.5%); A and D were most common in Uganda (57.5% and 38.4%, respectively) and Kenya (67.1% and 12.6%, respectively); and A/G (59.1%) and G (29.6%) in Nigeria (table 1).

The overall prevalence of resistance was 5.6% (95% CI 4.6–6.7%), which included 2.5% (1.8–3.2) resistance associated with NRTIs, 3.3% (2.5–4.2) associated with NNRTIs, and 1.3% (0.8–1.8) associated with protease inhibitors (figure 2, table 2, and webappendix table 1). Of the 139 sequences with at least one drug-resistance mutation, resistance was confined to a single drug-class for 112 sequences (80.6%), and 104 sequences (74.8%) had a single mutation. Dual-class resistance to NRTIs and NNRTIs was uncommon (25 sequences; sample-weighted proportion 1.2%, 95% CI 0.7–1.7), and triple-class resistance was rare (two sequences; 0.1%, 0.0–0.3). The most common drug-resistance mutations for NNRTIs were K103N, Y181C/I, and G190A/S; for NRTIs, the most common mutations were thymidine analogue mutations (TAMs) and M184V. Of the TAMs, M41L was identified most, then T215F/Y, K70R, and D67N and K219E (table 2). The K65R mutation, associated with cross-resistance to the NRTI class, was noted in one participant from Kampala. In the protease gene a high frequency of various naturally occurring polymorphisms was recorded, but few significant drug-resistance mutations, the most common being M46I/L and L90M (table 2). Of the 139 sequences with at least one drug-resistance mutation, 84 (59.2%, 95% CI 50.0–68.3) had high-level resistance (Stanford

Table 1. Baseline characteristics

Country		Zambia		South Africa			Uganda		Kenya		Zimbabwe	Nigeria
	Total (n=2590)	Lusaka* (n=555)	Pretoria (n=182)	Johannesburg (n=195)	White River (n=215)	Kampala (n=188)	Fort Portal (n=195)	Mbale (n=220)	Mombasa (n=210)	Nairobi (n=213)	Harare (n=213)	Lagos (n=204)
Site type		PFP, NGO, FBO	PFP	Public	NGO	Public	Public	Public	Public	FBO	NGO	Public
Year of start of ART rollout		2003	2004	2004	2004	2000	2002	2001	2003	2003	2004	2002
Enrolment period (month/year)		03/07-09/08	05/07-07/08	09/07-11/08	11/07-06/08	01/08-06/08	01/08-10/08	02/08-09/08	10/07-07/08	02/08-06/09	09/08-09/09	09/08-07/09
Sex												
Female	1486 (57.4)	300 (54.1)	93 (51.1)	145 (74.4)	127 (58.5)	100 (53.2)	107 (54.9)	116 (52.7)	126 (60.0)	114 (53.5)	137 (63.9)	124 (60.8)
Male	1104 (42.6)	255 (46.0)	89 (48.9)	50 (25.6)	90 (41.5)	88 (46.8)	88 (44.1)	104 (47.3)	84 (40.0)	99 (46.5)	77 (36.2)	80 (39.2)
Mean age, yrs (SD)	38.0 (9.0)	38.0 (9.1)	38.7 (8.5)	36.6 (7.4)	39.0 (10.1)	36.5 (8.9)	37.7 (10.3)	38.7 (9.6)	37.4 (8.6)	38.8 (8.7)	38.9 (8.8)	37.1 (8.5)
WHO clinical stage												
1 or 2	1015 (39.2)	251 (45.2)	71 (39.0)	125 (64.1)	57 (26.5)	70 (37.2)	70 (35.9)	44 (20.0)	79 (37.6)	63 (29.6)	100 (47.0)	85 (41.7)
3 or 4	1575 (60.8)	304 (54.8)	111 (61.0)	70 (35.9)	158 (73.5)	118 (62.8)	125 (64.1)	176 (80.0)	131 (62.4)	150 (70.4)	113 (53.1)	119 (58.3)
Median CD4 count, cells/μL (IQR) †	133 (62-204)	131 (63.5-198.5)	140 (50.5-229.5)	94.5 (28.8-160.3)	94.5 (35-154)	130.5 (49.3-211.8)	177 (94.5-259.5)	114 (50-178)	130.5 (63.5-194)	165 (106-224)	186 (112.5-259.5)	134 (68-200)
Mean HIV-RNA, $\log_{10}$ c/ml (SD) ‡	4.90 (1.0)	4.95 (0.9)	4.58 (1.0)	4.60 (1.0)	4.89 (0.8)	5.37 (0.8)	5.20 (0.9)	5.50 (0.8)	4.67 (0.9)	4.54 (0.9)	4.56 (1.2)	4.96 (1.0)

Table 1 (continued)

Country		Zambia		South Africa			Uganda			Kenya		Zimbabwe	Nigeria
	Total (n=2590)	Lusaka* (n=555)	Pretoria (n=182)	Johannesburg (n=195)	White River (n=215)	Kampala (n=188)	Fort Portal (n=195)	Mbale (n=220)	Mombasa (n=210)	Nairobi (n=213)	Harare (n=213)	Lagos (n=204)
HIV-1 subtypes §												
A	609 (25.0)	3 (0.6)	1 (0.6	1 (0.6)	0	100 (55.9)	102 (56.0)	126 (60.3)	131 (64.2)	140 (70.0)	0	5 (2.7)
C	1323 (54.3)	513 (97.7)	170 (96.6)	173 (97.2)	207 (100.0)	7 (3.9)	5 (2.8)	4 (1.9)	25 (12.3)	28 (14.0)	189 (99.5)	2 (1.1)
D	275 (11.3)	1 (0.2)	2 (1.1)	0	0	70 (39.1)	73 (40.1)	76 (36.4)	28 (13.7)	23 (11.5)	0	2 (1.1)
G	64 (2.6)	3 (0.6)	0	0	0	1 (0.6)	0	0	2 (1.0)	2 (1.0)	1 (0.5)	55 (29.6)
A/G	115 (4.7)	3 (0.6)	0	1 (0.6)	0	0	1 (0.6)	0	0	0	0	110 (59.1)
Other	50 (2.1)	2 (0.4)	3 (1.7)	3 (1.7)	0	1 (0.6)	1 (0.6)	3 (1.4)	18 (8.8)	7 (3.5)	0	12 (6.5)

Data are number of participants (%) unless otherwise stated. NGO, non-governmental organization; FBO, faith-based organization; PFP, private for profit; ART, antiretroviral therapy. *Combines participants from three clinical sites in Lusaka. †Data for n=2580. ‡Data for n=2563. §Data for n=2436.

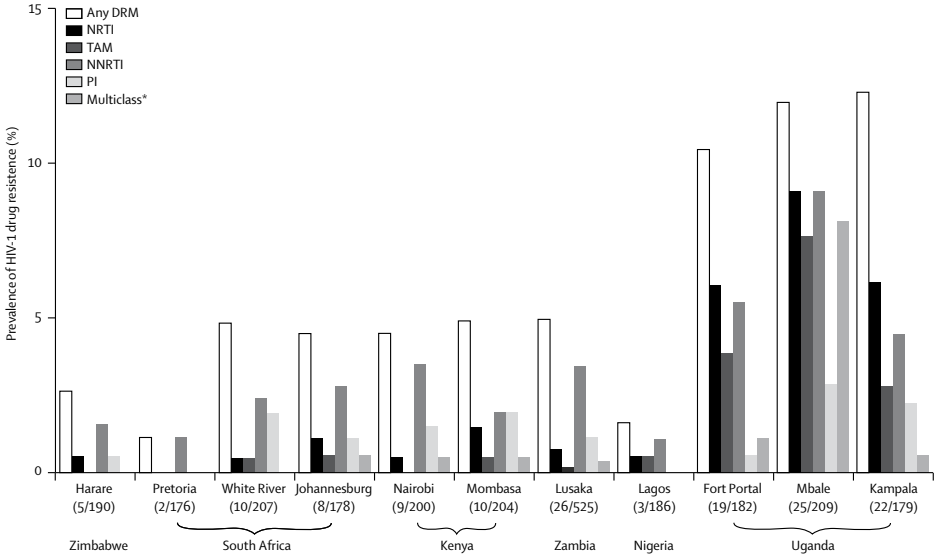

Figure 2. Prevalence of HIV-1 primary drug-resistance in antiretroviral-naive individuals in the PASER-M cohort, by region and drug class

People with at least one drug-resistance mutation shown as proportion of all people by region and drug class. Regions are clustered by country and sorted by descending calendar year of rollout of antiretroviral therapy. NRTI, nucleoside reverse transcriptase inhibitor; NNRTI, non-NRTI; PI, protease inhibitor; TAM, thymidine analogue mutation. * Multiclass, resistance to at least two drug classes

5), 25 (17.4%, 10.5–24.2) had intermediate-level resistance (Stanford 4), and 30 (23.5%, 15.5–31.4) had low-level resistance (Stanford 3). 84 sequences (60.1%, 95% CI 50.9–69.3) showed loss of susceptibility to NNRTIs (nevirapine 60.1%, efavirenz 59.3%), 51 (41.4%, 32.1–50.7) to NRTIs (lamivudine or emtricitabine 23.7%, zidovudine 28.2%, stavudine 29.2%, tenofovir 13.6%), and 13 (6.1%, 2.4–11.4) to ritonavir-boosted protease inhibitors.

The prevalence of primary drug resistance varied substantially between regions, ranging from 1.1% (95% CI 0.0–2.7) in Pretoria, South Africa, to 12.3% (7.5–17.1) in Kampala, Uganda (figure 2 and webappendix table 1). The pooled prevalence for all three Ugandan sites—situated in east, west, and central Uganda—was 11.6% (66 of 570; 95% CI 8.9–14.2), compared with 3.5% (73 of 1866; 95% CI 2.5–4.5) for all other sites. In Mbale, east Uganda, an unexpectedly high proportion of participants harboured the M184V (17 of 209; 8.1%, 95% CI 4.4–11.8) and T215F/Y mutations (11 of 209; 5.3%, 2.2–8.3), whereas these mutations were uncommon at all other sites (webappendix table 2). The exclusion of the 17 Mbale participants who harboured M184V would reduce the overall resistance prevalence to 4.8% (122 of 2419; 95% CI 3.8–5.7), the prevalence of M184V to 0.3% (eight of 2419; 0.1–0.5) and of T215F/Y to 0.0% (none of 2419; 0.0–0.3), and the Mbale site-specific overall resistance prevalence would decrease from 12.0% (25 of 209; 7.6–16.4) to 4.2% (eight of 192; 1.4–7.0; webappendix table 2).

Table 2. Frequencies of primary drug-resistance mutations.

	N	Proportion (95% CI) of sequences carrying DRMs (n=139)	Proportion (95% CI) of all sequences (n=2436)
Any DRM	139	100.0	5.6 (4.6, 6.7)
NRTI			
Any	54	43.7 (34.4, 53.0)	2.5 (1.8, 3.2)
Any TAM*	33	28.0 (19.6, 36.3)	1.6 (1.0, 2.1)
≥2 TAMs	13	10.9 (5.0, 16.7)	0.6 (0.3, 1.0)
M41L	16	14.0 (7.5, 20.5)	0.8 (0.4, 1.2)
K65R	1	0.7 (0.0, 2.0)	0.04 (0.0, 0.1)
D67E/G/N	12	9.9 (4.4, 15.4)	0.6 (0.2, 0.9)
K70R	11	8.5 (3.4, 13.7)	0.5 (0.2, 0.8)
M184V†	25	21.4 (13.7, 29.2)	1.2 (0.7, 1.7)
T215F/Y‡	14	12.8 (6.4, 19.1)	0.7 (0.3, 1.1)
K219E/Q	8	6.2 (1.7, 10.7)	0.4 (0.09, 0.6)
NNRTI			
Any	83	59.3 (50.1, 68.5)	3.3 (2.5, 4.2)
≥2	16	9.8 (4.2, 15.3)	0.6 (0.2, 0.9)
K101E	11	8.3 (3.0, 13.6)	0.5 (0.2, 0.8)
K103N/S	46	31.8 (23.1, 40.4)	1.8 (1.2, 2.4)
Y181C/I	19	12.3 (6.1, 18.6)	0.7 (0.3, 1.1)
G190A/S	17	12.1 (6.0, 18.2)	0.7 (0.3, 1.0)
PI			
Any	31	22.4 (14.6, 30.2)	1.3 (0.8, 1.8)
M46I/L	9	6.8 (2.2, 11.3)	0.4 (0.1, 0.6)
L90M	7	3.1 (0.0, 6.3)	0.2 (0.0, 0.4)

Proportions and CIs calculated accounting for the sampling weights of the sites. The table lists all drug-resistance mutations (DRMs) from the 2009 WHO DRM list, identified in 0.3% or greater of all sequences. DRMs identified in less than 0.3% of sequences were T69D, K70E, L74I/V, V75M, F77L, Y115F, L100I, V106M, Y188C/L, L210W, P225H in reverse-transcriptase and L23I, L24I, D30N, I50L/V, I54T/V, N83D, I85V, N88D in protease. NRTI, nucleoside reverse transcriptase inhibitor; TAM, thymidine analogue mutation; NNRTI, non-NRTI; PI=protease inhibitor. *M41L, D67N, K70R, L210W, T215Y/F, K219Q/E. †Exclusion of Mbale site reduces M184V to a frequency of 8. ‡Frequency of individual mutations at codon 215: F 4; I 3; S 1; and Y 10.

For additional quality control, we sent the 17 electropherograms from Mbale, which contained several drug-resistance mutations, to the South African laboratory for repeat analysis by an experienced laboratory technician from whom the first result was masked. The inter-observer result was concordant for 95 drug-resistance mutation pairs and discordant for four pairs of wild-type and drug-resistance mutation mixtures (reverse transcriptase positions 41, 67, 190, and 210), yielding no evidence for a significant difference (McNemar $p>0.1$). Additionally, viral phylogenetic trees for each site showed that each sequence was from a different individual and there was no evidence of laboratory

carry-over contamination. The previous antiretroviral-drug status was unconfirmed for only two Mbale participants, because they had died.

In multivariate analyses, the OR for drug resistance associated with each additional year since the start of the ART roll-out in a region was 1.38 (95% CI 1.13–1.68; p=0.001; table 3). In four sensitivity analyses, done by including the 11 participants who disclosed previous antiretroviral drug use, excluding Mbale, excluding all participants harbouring M184V, and excluding Mbale and all participants harbouring M184V, the strength of the association with time since start of ART roll-out did not significantly change (range OR 1.26–1.41 for each additional year). For NNRTI-resistance, the OR for each additional year since start of ART roll-out was 1.35 (1.01–1.81; p=0.041).

Table 3. Demographic and clinical factors associated with primary HIV-1 drug resistance.

	Number of sequences	**Prevalence of HIVDR**	**Univariate analysis***			**Multivariate analysis†**		
	N	N (%)	OR	95% CI	p-value	OR	95% CI	p-value
Total	2436	139						
Sex								
Female	1388	70 (5.0)	Reference					
Male	1048	69 (6.6)	1.33	0.94, 1.87	0.106			
Age (years)	37.9 (9.04)‡	38.3 (9.39)‡	1.01§	0.99, 1.02	0.591			
WHO clinical stage								
I	355	14 (3.9)	Reference					
II	582	38 (6.5)	1.70	0.91, 3.19	0.097			
III	1087	63 (5.8)	1.50	0.83, 2.71	0.180			
IV	412	24 (5.8)	1.51	0.77, 2.96	0.234			
CD4 count (cells/µl)	131 (61-202)¶	122 (44-181)¶	0.85 ‖	0.70, 1.04	0.108			
HIV RNA (log10 c/ml)	5.01 (0.81)‡	5.16 (0.78)‡	1.03**††	0.82, 1.30	0.777††			
Probable mode of infection								
Heterosexual contact	1620	103 (6.4)	Reference					
Other exposures‡‡	17	1 (5.9)	0.92	0.12, 7.01	0.936			
Unknown	799	35 (4.4)	0.67	0.46, 1.00	0.050			
HIV-1 subtype								
A	609	43 (7.1)	Reference			Reference		
C	1232	54 (4.1)	0.56	0.37, 0.85	0.006	1.08	0.59, 1.95	0.808
D	275	35 (12.7)	1.92	1.20, 3.07	0.007	1.61	0.99, 2.60	0.053
G	64	2 (3.1)	0.42	0.10, 1.79	0.244	0.48	0.11, 2.12	0.330

Table 3 (continued)

	Number of sequences	Prevalence of HIVDR	Univariate analysis*			Multivariate analysis†		
	N	N (%)	OR	95% CI	p-value	OR	95% CI	p-value
A/G	115	1 (0.9)	0.12	0.02, 0.85	0.034	0.13	0.02, 0.98	0.048
Other	50	4 (8.0)	1.14	0.39, 3.33	0.804	1.78	0.59, 5.37	0.308
Performance status§§								
Optimum	1240	51 (4.11)	Reference			Reference		
Reduced	974	82 (8.4)	2.14	1.50, 3.07	<0.001	1.68	1.12, 2.53	0.012
Sector								
Non-government	1298	52 (4.0)	Reference					
Public	1138	87 (7.6)	1.98	1.39, 2.82	<0.001			
Calendar year of sampling								
2007	581	28 (4.8)	Reference			Reference		
2008	1511	99 (6.6)	1.38	0.90, 2.13		0.64	0.36, 1.13	0.121
2009	344	12 (3.5)	0.71	0.36, 1.42		0.56	0.26, 1.21	0.143
Time since ART rollout in region (years)	4.68 (3.98-5.97)¶	5.65 (4.17-7.14)¶	1.38§	1.22, 1.56	<0.001	1.38§	1.13, 1.68	0.001

*Univariate logistic regression. †Multilevel multivariate logistic regression analysis. ‡Mean (SD). §OR per year. ¶Median (IQR). ||OR per 100 cells per µL CD4 count increase (nine values missing). **OR per 1 $\log_{10}$ copies per mL HIV-1 RNA increase. ††Adjusted for HIV-1 RNA assay. ‡‡Includes recipients of blood products, homosexual contact, and perinatal transmission. §§WHO performance scale (222 values missing). HIVDR, HIV-1 drug resistance; OR, odds ratio; ART, combination antiretroviral therapy.

Additionally, the association of any resistance with subtype was of marginal significance, with risk increased for D and reduced for A/G, compared with subtype A (table 3). Risk of resistance was increased for individuals who had a reduced performance status (table 3). Any resistance and NNRTI-resistance were not associated with sex, age, type of exposure, viral load, and site administration, including no apparent association with CD4 cell count and WHO stage—both markers of duration of infection. We did not identify significant interactions between region, calendar year of sampling, subtype, and time since ART roll-out.

DISCUSSION

Prevalence of primary drug resistance in antiretroviral-naive individuals is substantially higher in Uganda, where antiretroviral drugs were first available, compared with other African countries. Resistance is mostly confined to a single drug-class, most commonly NNRTIs. The spectrum of the major drug-resistance mutations in non-B subtype infected

Africans is largely similar to those known from studies in people infected with subtype B in developed countries [5,7-10]. The risk of primary resistance in a region rose by 38% for each additional year that elapsed since the start of the local ART roll-out. We confirmed the validity of this association in sensitivity analyses that eliminated possible effects of a single site (Mbale) and undisclosed previous antiretroviral-drug exposure (M184V mutation). The strengths of our study were its large international sample of patients, representing pretreatment populations in routine clinical practice, and the use of standardised data collection and measurement methods, which allowed for comparison across sites.

Our study provided the opportunity to make direct comparisons between regions and countries, investigating the effect of the timing of introduction of ART, as a proxy for the amount of circulating drug-resistant HIV-1 strains at the population level, on the level of primary resistance. Since populations can differ in other aspects, separation of the effects of the exposure alone is difficult. However, no evidence exists of other major differences between countries in terms of virological failure rates, drug regimens used, adherence levels, or drug supply continuity, which could provide an alternative explanation for our recorded differences in the prevalence of primary resistance. One exception is South Africa, which is the only country that has included routine viral-load monitoring in its national ART programme [30]. Our findings, therefore, support the hypothesis that the widespread distribution of ART in Africa is driving the emergence of primary drug resistance; this has important implications for public health, given that options for alternative treatment regimens are restricted in most settings. A limitation in this respect is that our results cannot be extrapolated outside the range of our measured exposure levels of 3–8 years since start of ART roll-out.

Our findings in Uganda accord with two recent reports (panel) that suggest rising levels of transmitted resistance in the region [16, 17]: from 3% (2005–06) to 7% (2007–08) in 408 people recently infected with HIV in east and southern Africa [16], and from 0% (2006–07) [31] to 8·6% (2009–10) in 70 newly diagnosed people in Kampala, Uganda [17]. After the limited-scale distribution of life-saving antiretroviral drugs in and around Kampala since the mid-1990s [32] access to ART became more widely available nationally since 2000–01, which contrasts with other countries in the region, which started scaling up ART since late 2003 and 2004. Therefore, other countries might anticipate an increase in primary resistance in coming years, similar to Uganda.

Drug-resistance mutations associated with NNRTIs were most common, which is consistent with the widespread use of this drug class as part of standard first-line ART, as well as single-dose nevirapine for prevention of mother to child transmission [33], the low

Panel: Research in context

Systematic review

We searched PubMed for English-language studies on primary antiretroviral-drug resistance in sub-Saharan Africa, published in 2001–11 with the MeSH terms "viral drug resistance","HIV-1", and "sub-Saharan Africa". Of 147 search results, we identified 38 eligible studies from 23 African countries (supplementary table 3). The comparability of most early studies was limited by differences in study populations, time periods, and definitions of drug resistance. Several WHO surveys of people newly diagnosed with HIV-1 estimated transmitted resistance to be low (<5%)[14]. Two recent reports suggested rising levels of transmitted resistance: from 3% (2005–06) to 7% (2007–08) in 408 people recently infected with HIV in east and southern Africa [16], and from 0% (2006–07) [31] to 8.6% (2009–10) in 70 newly diagnosed people in Kampala, Uganda [17].

Interpretation

Our observational multicentre study in six African countries is the first study in Africa we know of to clearly show an association between the time since start of ART roll-out in a region and the prevalence of primary drug-resistance. Our findings support the hypothesis that the ART roll-out in Africa is driving the emergence of primary drug-resistance. Resistance surveillance and prevention should be prioritized in settings where ART programmes are scaled up.

genetic barrier for the development of resistance to this class [34], the high prevalence noted in treated patients [13], persistence because of restricted fitness cost [35], and other local reports of primary resistance [16, 17]. The prevalence of NNRTI-resistant strains was not higher in women than in men, despite the possibility of undisclosed previous use of maternal prophylaxis to prevent mother-to-child transmission in women. However, this does not rule out the possibility of substantial onward transmission of these strains to men, and our study design did not allow a quantification of the extent to which the use of single-dose nevirapine contributed to the levels of primary resistance. TAMs, related to the extensive use of zidovudine and stavudine as part of first-line regimens [36], were more common in Uganda than in the other countries. In addition to the early ART roll-out in Uganda, we speculate that the restricted use of non-potent mono and dual regimens of thymidine analogues in Uganda before potent ART became available [32], might have contributed to the circulation of TAMs, as happened in developed countries [5, 7, 8].

The validity of measuring resistance in antiretroviral-naive, chronically infected populations is debatable for two main reasons. Because our study population was probably infected on average several years earlier, the detected primary resistance patterns relate to the lower availability of antiretroviral drugs and consequently fewer circulating drug-resistant strains in the past. Although transmitted drug-resistant variants might persist for a couple of years in antiretroviral-naive individuals [35, 37], chronic infection provides the opportunity for reversion to wild-type virus or diminution to levels below detection by population-based genotyping [38]. This reversion or diminution might result in an underestimation of the prevalence of primary resistance. Second, some of the

detected drug-resistance mutations might be acquired because of undisclosed previous antiretroviral-drug exposure. Through targeted efforts, we excluded an additional 11 of 107 individuals who disclosed previously unreported exposure to ART, post-exposure prophylaxis, or drugs given for prevention of mother-to-child transmission. On the basis of this finding, we can estimate that, of the 43 individuals who had an undetermined previous antiretroviral drug status, we might have missed another four antiretroviral-exposed individuals. The possibility of undisclosed exposure to ART was highlighted in Mbale, where we recorded an unexpectedly high frequency of the M184V mutation.

Nonetheless, given the challenges of identifying individuals during recent HIV infection, we argue that there is value in surveying resistance in populations starting ART. Particularly, resistance data in pretreatment populations provide important information about the probable effectiveness of available regimens for each region. Our study population comprised mostly free-access, regular ART programmes in urban areas where massive ART programmes have been implemented in recent years. However, our sites were not necessarily representative of all people with HIV/AIDS in their respective countries and caution is warranted when extrapolating results to different subpopulations, countries, or rural areas.

Prevalence of resistance was higher in subtype D than in subtype A. Given the lack of evidence for clinically relevant differences between subtypes with regard to the genetic barrier to resistance [39] and clinically significant drug-resistance mutations [18] and that subtype is highly correlated with region, the differential risk between subtypes is most probably confounded by differences in antiretroviral-drug selective pressure between regions. Other potential determinants of resistance transmission, especially accurate data on the route, time, and source of infection, and the source's antiretroviral drug history, were not available for our analysis.

In conclusion, we showed an association between the duration of ART availability in African settings and the level of primary HIV-1 drug-resistance. The high primary resistance prevalence that we identified in Uganda, where antiretroviral drugs were first available, presents an unequivocal warning to other nearby countries. Future treatment guidelines in Africa should take into account the local levels of primary resistance. Further studies are needed to establish the cost-effectiveness of extended genotypic resistance testing in Africa. Our results provide a basis for repeated epidemiological studies to measure the population effects of HIV-treatment programmes over time.

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Contributors

TFRW conceived the study and was the principal investigator. RLH, CLW, WSS, RS, MvV, and TFRW designed the study and developed the protocol. RLH, KCES, and MvV contributed to implementation. CK, MS, FC, MB, KM, MW, and AO established the cohort and supervised data collection. CLW, IN, and WSS conducted and supervised the laboratory testing. RLH conducted the sequence and statistical analyses and drafted the manuscript. FWW checked and supervised the statistical analyses. CLW, KCES, RS, FWW, and TFRW provided input to interpretation of the data and critically reviewed the paper for important intellectual content. All authors reviewed and approved the final version.

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WEBAPPENDIX

Panel. GenBank accession numbers

All HIV-1 *pol* sequences in this study have been deposited in GenBank under the following accession numbers:

HM119603, HM119605-HM119660, HM119662-HM119669, HM119671-HM119682, HM119684-HM119689, HM119691-HM119698, HM119700-HM119715, HM119717-HM119718, HM119720, HM119722-HM119725, HM119728-HM119736, HM119738-HM119760, HM119762-HM119795, HM119797-HM119851, HM119853-HM119855, HM119857-HM119869, HM119871-HM119971, HM119973-HM119982, HM119985-HM120021, HM120023-HM120054, HM120056, HM120058-HM120073, HM120075-HM120089, HM120091-HM120128, HM120130-HM120146, HM120148-HM120150, HQ993572- HQ995497

Table 1. Prevalence of primary HIV-1 drug resistance in the PASER-M cohort, by region and drug class.

	Sequences	Total HIVDR		NRTI						NNRTI		PI		NRTI+ NNRTI		NRTI+ NNRTI +PI	
				Any		TAM		M184V									
	N	N	%	N	%	N	%	N	%	N	%	N	%	N	%	N	%
Zambia																	
Lusaka [a]	525	26	5.0	4	0.8	1	0.2	2	0.4	18	3.4	6	1.1	2	0.4	0	0.0
South Africa																	
Pretoria	176	2	1.1	0	0.0	0	0.0	0	0.0	2	1.1	0	0.0	0	0.0	0	0.0
Johannesburg	178	8	4.5	2	1.1	1	0.6	1	0.6	5	2.8	2	1.1	1	0.6	0	0.0
White River	207	10	4.8	1	0.5	1	0.5	0	0.0	5	2.4	4	1.9	0	0.0	0	0.0
Uganda																0	0.0
Kampala	179	22	12.3	11	6.2	5	2.8	1	0.6	8	4.5	4	2.2	1	0.6	0	0.0
Fort Portal	182	19	10.4	11	6.0	7	3.9	2	1.1	10	5.5	1	0.6	2	1.1	0	0.0
Mbale	209	25	12.0	19	9.1	16	7.7	17	8.1	19	9.1	6	2.9	17	8.1	2	1.0
Mbale [b] (excluding M184V)	192	8	4.2	2	1.0	1	0.5	-	-	2	1.0	1	0.6	0	0.0	0	0.0
Kenya																0	0.0
Mombasa	204	10	4.9	3	1.5	1	0.5	1	0.5	4	2.0	4	2.0	1	0.5	0	0.0
Nairobi	200	9	4.5	1	0.5	0	0.0	1	0.5	7	3.5	3	1.5	1	0.5	0	0.0
Zimbabwe																0	0.0
Harare	190	5	2.6	1	0.5	0	0.0	0	0.0	3	1.6	1	0.5	0	0.0	0	0.0
Nigeria																0	0.0
Lagos	186	3	1.6	1	0.5	1	0.5	0	0.0	2	1.1	0	0.0	0	0.0	0	0.0
Total HIVDR [c]	2436	139	5.6	54	2.5	33	1.6	25	1.2	83	3.3	31	1.3	25	1.2	2	0.1

HIVDR, HIV-1 drug resistance; NRTI, nucleoside reverse transcriptase inhibitor; NNRTI, non-NRTI; PI, protease inhibitor; TAM, thymidine analogue mutation.

[a] Combines participants from three clinical sites in Lusaka.

[b] Resistance prevalence in Mbale site after excluding sequences carrying the M184V mutation.

[c] Overall proportions accounting for the sampling weights of the sites.

Table 2. Frequencies of primary drug-resistance mutations in PASER-M cohort, by region.

	N	Lusaka[a]	Pretoria	Johannesburg	White River	Kampala	Fort Portal	Mbale	Mombasa	Nairobi	Harare	Lagos
Total no. sequences	2436	525	176	178	207	179	182	209	204	200	190	186
Total no. DRMs	245	37	2	14	12	25	26	97	12	11	5	4
NRTI - Total	111	5	0	6	1	13	14	65	4	1	1	1
M41L	16				1	3	4	7				1
K65R	1					1						
D67E/G/N	3/1/8			1/0/1		1/0/1		0/1/6	1/0/0			
T69D	4	1				1		1			1	
K70E/R	1/11	0/1		0/1		0/1	0/2	1/6				
L74I/V	3/1					0/1		2/0	1/0			
V75M	3					1		2				
F77L	1						1					
Y115F	1						1					
M184V	25	2		1		1	2	17	1	1		
L210W	6						1	5				
T215F/I/S/Y	4/3/1/10			1/0/0/0		0/1/1/0	0/2/0/1	3/0/0/8	0/0/0/1			
K219E/Q	3/5	1/0		1/0				1/5				
NNRTI - Total	**102**	**26**	**2**	**6**	**7**	**8**	**11**	**25**	**4**	**7**	**3**	**3**
L100I	3	2							1			
K101E/P	11/0	2/0		1/0		3/0		3/0		1/0		1/0
K103N/S	43/3	7/3	1/0	3/0	5/0	2/0	8/0	9/0	3/0	3/0	1/0	1/0
V106A/M	0/2	0/1								0/1		
Y181C/I/V	18/1/0	6/0/0	1/0/0	1/0/0		2/0/0	1/0/0	4/1/0		2/0/0		1/0/0
Y188C/L	1/1	1/0									0/1	
G190A/S	16/1	3/1		1/0	1/0		2/0	8/0			1/0	
P225H	2				1	1						
PI - Total	**32**	**6**	**0**	**2**	**4**	**4**	**1**	**7**	**4**	**3**	**1**	**0**
L23I	1							1				
L24I	2					2						
D30N	1							1				
M46I/L	4/5			0/1	4/0			0/1	0/3			
I50L/V	2/0	1/0						1/0				
I54T/V	1/1					0/1		1/0				
N83D	1					1						
I85V	6	1						1	1	2	1	
N88D	1						1					
L90M	7	4		1				1		1		

DRM, drug-resistance mutation; NRTI, nucleoside reverse transcriptase inhibitor; NNRTI, non-NRTI; PI, protease inhibitor.

[a] Combines participants from three clinical sites in Lusaka.

Table 3. Systematic review of studies that measured the prevalence of primary drug resistance in HIV-1 infected persons in sub-Saharan Africa.

Angola								
Castelbranco *et al.* (1)	35	2008-2009	Newly diagnosed	WHO	5.7%	1	2	0
Botswana								
Bussmann *et al.* (2)	71	2001	Drug-naïve	n/a	0.0%	0	0	0
Bussmann *et al.* (3)	71	2007	Newly diagnosed	WHO	0.0%	0	0	0
Burkino Faso								
Vergne *et al.* (4)	97	2003	Drug-naïve	IAS-USA	8.2%	3	4	2
Tebit *et al.* (5)	104	2004-2006	Drug-naïve	IAS-USA	12.5%	11	6	0
Ayouba *et al.* (6)	51	2005	Newly diagnosed	WHO	0.0%	0	0	0
Burundi								
Vidal *et al.* (7)	101	2002	Drug-naïve	IAS-USA	1.0%	0	1	0
Cameroon								
Vergne *et al.* (4)	102	2001-2002	Drug-naïve	IAS-USA	7.8%	3	2	3
Soares *et al.* (8)	59	2002-2005	Drug-naïve	WHO	5.1%	1	0	3
Ndembi *et al.* (9)	75	2004	Newly diagnosed	IAS-USA	14.7%	6	7	2
Aghokeng *et al.* (10)	47	2006-2007	Newly diagnosed	WHO	6.4%	1	2	0
Aghokeng *et al.* (10)	44	2006-2007	Newly diagnosed	WHO	6.8%	2	1	0
Central African Republic								
Marechal *et al.* (11)	117	2005	Drug-naïve	IAS-USA	0.0%	0	0	0
Chad								
Aghokeng *et al.* (10)	34	2006-2007	Newly diagnosed	WHO	0.0%	0	0	0
Congo DR								
Vidal *et al.* (12)	70	2002	Drug-naïve	IAS-USA	4.3%	0	1	2
Cote d'Ivoire								
Toni *et al.* (13)	107	2001-2002	Drug-naïve	IAS-USA	5.6%	1	4	1
Toni *et al.* (14)	100	2002-2006	Newly diagnosed	IAS-USA	6.0%	3	3	1
Ayouba *et al.* (6)	48	2007	Newly diagnosed	WHO	0.0%	0	0	0
Djibouti								
Maslin *et al.* (15)	47	2002	Drug-naïve	ANRS algorithm	21.2%	1	7	3
Equatorial Guinea								
Djoko *et al.* (16)	41	2008	Drug-naïve	WHO	4.9%	1	0	1
Ethiopia								
Kassu *et al.* (17)	92	2003	Drug-naïve	IAS-USA	3.3%	1	2	0
Kenya								
Price *et al.* (18)	64	2006-2009	Recently infected	WHO	3.1%	1	1	0
Lihana *et al.* (19)	53	2005	Drug-naïve	IAS-USA	7.5%	5	6	0
Malawi								
Kamoto *et al.* (20)	34	2006	Newly diagnosed	WHO	0.0%	0	0	0

Table 3 (continued)

Mali								
Derache *et al.* (21)	98	2005	Drug-naïve	IAS-USA	2.0%	1	1	0
Haidara *et al.* (22)	101	2007-2008	Drug-naïve	Virco algorithm	9.9%	9	6	0
Mozambique								
Abreu *et al.* (23)	75	2002	Drug-naïve	IAS-USA	0.0%	0	0	0
Bartolo *et al.* (24)	68	2002-2004	Drug-naïve	IAS-USA	5.9%	12	1	0
Bellochi *et al.* (25)	58	2003	Drug-naïve	IAS-USA	0.0%	0	0	0
Rwanda								
Price *et al.* (18)	78	2006-2009	Recently infected	WHO	7.7%	0	6	1
Senegal								
Ndiop-Ndiaye *et al.* (26)	96	1998-2001	Drug-naïve	WHO	4.2%	4	0	1
Ndiop-Ndiaye *et al.* (26)	104	2003-2007	Drug-naïve	WHO	1.9%	0	0	2
Ayouba *et al.* (6)	48	2007	Newly diagnosed	WHO	0.0%	0	0	0
South Africa								
Gordon *et al.* (27)	72	2001-2002	Drug-naïve	n/a	2.8%	0	3	0
Bessong *et al.* (28)	35	2001-2004	Drug-naïve	IAS-USA	1.0%	1	0	0
Pillay V *et al.* (29)	65	2002	Drug-naïve	WHO	0.0%	0	0	0
Jacobs *et al.* (30)	140	2002-2004	Drug-naïve	IAS-USA	2.1%	0	3	0
Pillay V *et al.* (29)	48	2004	Drug-naïve	WHO	4.2%	2	0	0
Huang *et al.* (31)	425	2006	Drug-naïve	IAS-USA	2.3%	3	7	2
Tanzania								
Somi *et al.* (32)	39	2005-2006	Newly diagnosed	WHO	0.0%	0	0	0
Togo								
Yaotse *et al.* (33)	75	2006-2007	Drug-naïve	WHO	10.7%	6	1	1
Uganda								
Eshleman *et al.* (34)	104	1998-2003	Recently infected	Geneseq	5.8%	3	2	3
Ndembi *et al.* (35)	37	2006-2007	Newly diagnosed	WHO	0.0%	0	0	0
Ndembi *et al.* (36)	70	2009-2010	Newly diagnosed	WHO	8.6%	3	2	1
Price *et al.* (18)	89	2006-2009	Recently infected	WHO	6.7%	1	2	3
Zambia								
Gonzalez *et al.* (37)	30	1998-2002	Newly diagnosed	WHO	0.0%	0	0	0
Gonzalez *et al.* (37)	86	2005	Newly diagnosed	WHO	2.3%	1	1	0
Price *et al.* (18)	169	2006-2009	Recently infected	WHO	2.4%	3	2	0
Hamers *et al.* (38)	523	2007-2008	Drug-naïve	WHO	5.2%	5	19	6

Studies are sorted by country and by ascending calendar year of sampling.
WHO, World Health Organization mutation list; IAS-USA, International Antiviral Society-USA mutation list; ANRS, Agence Nationale de Recherches sur le Sida et les hépatites virales; NRTI, nucleoside reverse transcriptase inhibitor. NNRTI, non-NRTI; PI, protease inhibitor.

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AFRIKA

Chapter 7

Transmitted antiretroviral drug resistance among newly HIV-1 diagnosed young individuals in Kampala

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ABSTRACT

Objective

To assess the emergence of transmitted HIV-1 drug resistance (TDR) in Kampala, Uganda, ten years after the scale-up of antiretroviral treatment (ART), and to compare with a previous survey among antenatal clinic attendees in 2007 (reporting 0% TDR).

Design

A cross-sectional survey was conducted among newly HIV-1 diagnosed, antiretroviral-naive young adults attending two large voluntary counselling and testing centers within the geographic area of Kampala.

Methods

Proxy criteria for recent HIV-1 infection were used as defined by the World Health Organization. Population sequencing of the *pol* gene was performed on plasma samples with HIV-1 RNA ≥1000 copies/mL. Drug resistance mutations (SDRMs) were identified according to the 2009 World Health Organization list for surveillance of TDR. HIV-1 subtypes were designated using maximum likelihood phylogenetic reconstruction.

Results

Genotypic test results were obtained for 70 of 77 (90.9%) participants. SDRMs were identified in six samples yielding a prevalence of TDR of 8.6% (95% confidence interval 3.2% to 17.7%). Two had SDRMs to nucleoside reverse-transcriptase inhibitors (D67G, L201W), three had SDRMs to non-NNRTIs (G190A, G190S, K101E), and one had SDRMs to protease inhibitors (N88D). Frequencies of HIV-1 subtypes were: A (36/70, 51.4%), C (2/70; 2.9%), D (23/70, 32.9%) and unique recombinant forms (9/70, 12.9%).

Conclusions

This repeated survey suggests an increase of TDR in Kampala, compared with a previous survey. This finding justifies increased vigilance with respect to surveillance of TDR in areas in Africa where ART programs are rolled-out.

INTRODUCTION

Expanded access to combination antiretroviral therapy (ART) in many countries in sub-Saharan Africa during the past decade [1] has remarkably improved the prognosis of HIV-1 infected individuals [2]. Important deficiencies in health systems, such as lack of virological monitoring and intermittent drug supply, have raised concerns about the rapid emergence and spread of drug-resistant HIV-1 strains in Africa [3, 4]. Increasing levels of transmitted drug-resistant HIV-1 variants (TDR) could compromise the effectiveness of standard first-line ART regimens [5, 6], which has severe public health consequences in areas where treatment options are limited. With the wider use of ART in industrialized countries, TDR to non-nucleoside reverse transcriptase inhibitors (NNRTIs) in newly infected individuals steadily increased, in San Francisco from 0% in 1996-1997 to 13.2% in 2000-2001 [5] and in Europe from 2.3% in 1996–1998 to 9.2% in 2001–2002 [6]. Genotypic resistance to two or more classes of antiretroviral (ARV) drugs increased from 2.5% to 13.2% [5].

Uganda was among the first African countries to distribute life-saving ARV medication. By the end of September 2009, nationwide an estimated 200,413 patients were receiving ART, reaching 39% of those in need [1]. In the capital city of Kampala the massive scale-up of ART was initiated in the year 2000, following limited-scale distribution since the mid 1990s. A survey performed in Entebbe, situated in the greater Kampala area, in 2006-2007 did not detect any significant drug-resistance mutations among 47 newly HIV-1 diagnosed pregnant women with CD4 count > 350 cells/μL attending an antenatal clinic [7].

We report the results of a subsequent survey in 2009-2010 that evaluated the prevalence of TDR among newly HIV-1 diagnosed young individuals attending voluntary counselling and testing (VCT) sites in Kampala, Uganda.

METHODS

Study design and population

A cross-sectional survey was conducted among clients attending two large free-access, non-governmental VCT sites in Kampala, Uganda: AIDS Information Centre (AIC), situated in Mengo area, and Naguru Teenage Health Information Centre (NTC), situated in Bugolobi area. The institutional review boards at the Academic Medical Center and the Uganda Virus Research Institute approved the study. Mandatory eligibility criteria, as defined by the World Health Organization (WHO), were used to identify individuals who were likely to have been recently infected [9]: newly diagnosed with HIV-1 and aged

between 18 and 25 years, or laboratory evidence of recent HIV-1 infection (defined as a confirmed HIV-1 positive antibody test with a negative HIV-1 antibody test within the past 12 months, or an indeterminate/negative HIV-1 antibody test with detectable HIV-1 RNA or positive p24 antigen). Exclusion criteria were any previous ARV use (also for the prevention of mother-to-child transmission of HIV-1), documented WHO clinical stage 4 event and previous pregnancy (parity) [9]. All participants provided written informed consent prior to study enrolment. During the enrolment period of maximum 12 months, the VCT clients were all screened and sequentially enrolled. A case report form was completed and a blood draw was performed in all participants.

Laboratory procedures

Plasma was separated within two hours from blood draw and stored immediately at –80°C. HIV-1 RNA was tested with the Amplicor MONITOR 1.5 (Roche, Roche Molecular Systems, NJ, USA). HIV-1 RNA was extracted from 140µl of blood plasma using the Qiamp viral RNA mini kit (Qiagen Inc, Chatsworth, CA). Polymerase gene-specific primers were used for reverse transcriptase, followed by nested PCR to amplify a 1030-base pair *pol* gene encompassing amino acids 1–99 of protease and 1–242 of reverse transcriptase. The PCR products were then purified with a QIA-quick PCR purification kit (Qiagen, Valencia, CA) and sequenced in the sense and antisense direction with a set of nested primers [10]. To ensure the quality of the data set, each sequence was checked before inclusion using ViroScore Suite v8.1 (ABL, France).

Genotypic Resistance and Phylogenetic Analysis

Samples were sequentially genotyped and TDR was analyzed. Drug resistance mutations (SDRMs) were identified according to the 2009 WHO list for surveillance of genotypic TDR updated in 2009, which excludes polymorphisms [11]. For SDRM analysis, the Stanford calibrated population resistance analysis tool version 5.0 beta was used [12]. *Pol* region subtype classification and recombinant patterns were determined using the REGA subtyping tool [13] and the SCUEAL application [14], further confirmed using phylogenetic analysis. We performed maximum likelihood phylogenetic reconstruction using PhyML based on the General Time-Reversible model with gamma distributed rate variation among nucleotide sites.

Statistical methods

The survey sample size was estimated from the hypothesis that the prevalence of TDR in the target population was initially low (estimated at 2%) and increased with time (estimated at 10%). To detect such increase with 80% power using a two-sided significance level of 0.05, the required number of HIV-1 sequences per geographic area was 78. Assuming 10% amplification failure, the target sample was 85 individuals. The proportions

of sequences containing ≥1 SDRM were calculated overall and by each of the three main drug classes, i.e. protease inhibitors (PIs), nucleoside reverse transcriptase inhibitors (NRTIs) and NNRTIs. TDR prevalence was estimated with a 95% confidence interval (CI) based on the binomial distribution. As a secondary analysis, the WHO-recommended truncated sampling technique was used to categorize TDR prevalence as low (<5%), moderate (5–15%), or high (>15%) for each of the three drug classes, based on the testing of the first ≤ 47 sequences [15]. Categorical data were compared using Chi-square test. Continuous data were investigated using Kruskal-Wallis or Student t-test. All analyses were performed using Stata version 10 (StataCorp LP, TX).

RESULTS

Patient characteristics

Study enrolment took place from February 2009 to February 2010 at AIC and from May 2009 to May 2010 at NTC. A total of 884 individuals were screened, of whom 81 (9.2%) met the eligibility criteria. Excluding four individuals due to protocol violations (i.e. 3 did not meet the age criterion, 1 had a previous pregnancy), 77 participants (43 from AIC and 34 from NTC) were included in the analysis (table 1). Seventy-six participants qualified based on the age criterion and one participant had a new confirmed HIV-1 diagnosis after a recent negative test. The mean age was 21.6 years (standard deviation, SD 2.1). Females comprised 70.1% (n=54). The mean age was lower for females (21.1 years, SD 2.0), compared to males (22.7 years, SD 1.9, p=0.0017). The median CD4 count was 417 cells/μL (interquartile range (IQR): 318.5-551.5 cells/μL) and the median HIV-1 RNA load was 4.49 $\log_{10}$ copies/ml (IQR: 3.96-5.28 $\log_{10}$ copies/ml). 94.8% (n=73) of participants were Ugandan nationals. Nearly all (73, 94.8%) participants reported sexual encounters with the opposite sex, whereas other exposures were uncommon. The median age at sexual debut was 18 years, with a range between 14 and 27 years. During the three years prior, 71 (92.2%) participants reported to have engaged in unprotected sex, with an average of 2.1 (SD 1.8) sexual partners, and 23 (29.9%) reported a first episode of a sexually transmitted infection. Among participants who had a steady sexual partner, 68.8% was unaware of their partner's HIV-1 status. Baseline characteristics, except for mean age, did not differ between sites (table 1).

Genotypic profiles

70 samples were successfully genotyped and seven samples failed to amplify or had no valid genotype. One or more SDRMs were identified in six of the 70 valid sequences, yielding an estimated TDR prevalence of 8.6% with a 95% CI 3.2% to 17.7%. The proportion of sequences with SDRMs associated with NRTIs, NNRTIs and PIs was 2.9% (2/70),

Table 1. Patient characteristics, by study site

	Total	Aids Information Centre (AIC)	Naguru Teenage Health Information Centre (NTC)	p-value
Patients	77	43	34	
Sex				0.085
Female	54 (70.1)	27 (65.9)	27 (79.4)	
Male	23 (29.9)	16 (39.0)	7 (20.6)	
Age – mean yrs (sd)	21.6 (2.1)	22.4 (1.9)	20.7 (1.9)	<0.001
Ugandan nationality	73 (94.8)	41 (95.4)	32 (94.1)	0.809
Marital status				0.075
Now married/cohabiting	14 (18.2)	5 (11.6)	9 (26.5)	
Divorced/separated	0	0	0	
Widowed	4 (5.2)	4 (9.3)	0	
Never married/single	58 (75.3)	34 (79.1)	24 (70.6)	
Other	1 (1.3)	0	1 (2.9)	
Education level				0.134
None/illiterate	2 (2.6)	1 (2.3)	1 (2.9)	
Primary school	24 (31.2)	9 (20.9)	15 (44.1)	
Secondary school	30 (39.0)	18 (41.9)	12 (35.3)	
Higher education	21 (27.3)	15 (34.9)	6 (17.7)	
Main occupation				0.014
None/at home	24 (31.2)	7 (16.3)	17 (50.0)	
Student	16 (20.8)	9 (20.9)	7 (20.6)	
Employed	35 (46.7)	25 (61.0)	10 (29.4)	
Hemoglobin, median g/dL (IQR) [a]	12.6 (11.3-14.3)	13.1 (11.4-14.8)	12.4 (10.8-14.0)	0.1284
CD4 cell count, median cells/μL (IQR) [b]	417 (318.5-551.5)	377.5 (236-519)	418.5 (264-573)	0.1159
HIV RNA, median $\log_{10}$ c/ml (IQR)	4.49 (3.96- 5.28)	4.47 (3.90-5.03)	4.55 (3.83-5.27)	0.4447

Data represent n (%) unless otherwise specified; [a] Data available for n=73; [b] Data available for n=76.

4.3% (3/70) and 1.4% (1/70), respectively. We observed six different SDRMs: D67G, K101E, G190S, G190A and L210W in reverse-transcriptase and N88D in protease. TDR was confined to a single drug-class in all six sequences. Table 2 summarizes the demographic and virological characteristics of the six participants who harboured an SDRM.

Using the WHO-recommended truncated sequential sampling technique, four of the first 47 sequences harboured an SDRM (moderate prevalence category), of which two were NRTI-associated (low prevalence category), one PI-associated (low prevalence category); and one NNRTI-associated. HIV-1 subtype frequencies were: A (36/70, 51.4%), C (2/70; 2.9%), D (23/70, 32.8%), A1/D recombinants (9/70, 12.9%).

Table 2. Demographic and virological characteristics of the six participants who harboured a drug resistance mutation.

#	Date of enrolment	ID	Age (years)	Sex	Estimated year of infection	Estimated country of infection	CD4 count (cells/µl)	HIV RNA load (log_{10} c/ml)	Viral subtype	Surveillance Drug-Resistance Mutations		
										NRTI	NNRTI	PI
1	19-Mar-09	33	23	Female	Unknown	Unknown	615	3.84	D		G190S	
2	21-May-09	702	20	Female	2008	Uganda	348	5.88	A (A1)	L210W		
3	05-Jun-09	705	20	Male	2008	Uganda	706	4.60	A	D67G		
4	27-Jul-09	248	23	Male	Unknown	Uganda	457	5.55	A1D			N88D
5	06-Jan-10	438	24	Male	Unknown	Uganda	270	5.47	A (A1)		G190A	
6	25-Mar-10	728	24	Male	2009	Uganda	495	5.88	D		K101E	

NRTI, nucleoside reverse transcriptase inhibitor; NNRTI, non-NRTI; PI, protease inhibitor

DISCUSSION

This survey among 70 newly HIV-1 diagnosed young VCT clients in Kampala demonstrated an estimated prevalence of TDR of 8.6%, which is likely to represent an increase compared to the previous survey in 2006-2007 that did not detect any SDRMs among 47 pregnant women from the greater Kampala area [7]. Identified SDRMs were associated with NNRTIs (3), NRTIs (2) and PIs (1), but in each sequence TDR was confined to a single drug class. This study is among the first to suggest an increase of TDR between repeated surveys within the same geographic area in Africa, although the subsequent surveys targeted different subpopulations.

Most studies from Africa that were conducted during the early scale-up of ART have reported low levels of TDR [7- 9]. In Botswana, the 2007 threshold survey indicated that five years following the countrywide ART roll-out, TDR was still less than 5% [16]. The IAVI cohort, however, of newly HIV-1 infected individuals in east and southern Africa reported a 5% overall prevalence of TDR, with an increase from 3% (4/157) in 2005-2006 to 7% (12/169) in 2007-2008 [17]. The proportions of participants who harboured TDR was particularly high in Entebbe (4/17, 23.5%) and Kigali (8/68, 11.8%) [17]. Consistent with this report, our study supports the hypothesis that increasing ARV drug exposure in African populations, following the roll-out of ARVs for treatment and prevention of mother-to-child transmission, may cause a rise in TDR and thereby new public health challenges.

In this study the categorization of TDR using prevalence (6/70) corresponded with the WHO-recommended truncated sequential sampling technique (4/47), i.e. "moderate" overall and "low" for each drug class separately. It should, however, be noted that the small sample sizes resulted in a wide confidence interval, warranting caution in interpreting and extrapolating the results.

This study has several limitations. Given the challenges, especially in resource-limited settings, in identifying individuals during acute or recent HIV-1 infection, WHO recommends the use of proxy criteria for the surveillance of TDR. A recent study in Botswana, however, found poor agreement between the WHO criteria and two laboratory-based methods to detect new infection [16]. The WHO approach could therefore lead to the inclusion of individuals with established infection, during which drug-resistant mutants may have reverted to wild-type virus [20, 21], thereby possibly underestimating the true current prevalence of resistance transmission. Although the study specifically selected newly diagnosed, ARV-naïve individuals, it cannot be completely ruled out that some participants had unknown or undisclosed prior exposure to ARV therapy and/or prophylaxis.

In conclusion, ten years following the ART scale-up in Kampala, Uganda, this repeated survey demonstrated that 8.6% of newly HIV-1 diagnosed youth harboured TDR, which is likely to represent an increase compared to the previous survey. The study findings should trigger public health action in performing additional surveys in the upcoming years to evaluate the evolution of TDR in the country and can provide guidance to drug-resistance prevention strategies. This is especially urgent since current options for first-line therapy in Uganda are limited and access to second-line therapy is not widely available.

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Author contributions

N.N., R.L.H., C.K., P.K., and T.F.R.W. conceived and designed the study. K.C.E.S. and C.W. coordinated the field work. B.M. supervised data collection. N.N. supervised analyzed the resistance data, and performed phylogenetic analysis and B.N. and F.L. performed and interpreted the laboratory testing. R.L.H. analyzed the clinical data and the resistance data. N.N. and R.L.H. drafted the paper. K.C.E.S. and T.F.R.W. reviewed the paper for important intellectual content. All authors read and approved the final manuscript. T.F.R.W. is the guarantor.

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AFRIKA

Part II

Antiretroviral treatment and acquired drug resistance


AFRIKA

Chapter 8

Effect of pretreatment HIV-1 drug resistance on immunological, virological, and drug-resistance outcomes of first-line antiretroviral treatment in sub-Saharan Africa: a multicentre cohort study

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ABSTRACT

Background

The effect of pretreatment HIV-1 drug resistance on the response to first-line combination antiretroviral therapy (ART) in sub-Saharan Africa has not been assessed. We studied pretreatment drug resistance and virological, immunological, and drug-resistance treatment outcomes in a large prospective cohort.

Methods

HIV-1 infected patients in the PharmAccess African Studies to Evaluate Resistance Monitoring (PASER-M) cohort started non-nucleoside reverse transcriptase inhibitor-based ART at 13 clinical sites in six countries, from 2007 to 2009. We used the International Antiviral Society-USA drug resistance mutation list and the Stanford algorithm to classify participants into three pretreatment drug resistance categories: no pretreatment drug resistance, pretreatment drug resistance with fully active ART prescribed, or pretreatment drug resistance with reduced susceptibility to at least one prescribed drug. We assessed risk factors of virological failure (≥400 copies per mL) and acquired drug resistance after 12 months of ART by use of multilevel logistic regression with multiple imputations for missing data. CD4 cell count increase was estimated with linear mixed models.

Findings

Pretreatment drug resistance results were available for 2579 (94%) of 2733 participants; 2404 (93%) had no pretreatment drug resistance, 123 (5%) had pretreatment drug resistance to at least one prescribed drug, and 52 (2%) had pretreatment drug resistance and received fully active ART. Compared with participants without pretreatment drug resistance, the odds ratio (OR) for virological failure (OR 2.13, 95% CI 1.44–3.14; p<0.0001) and acquired drug-resistance (2.30, 1.55–3.40; p<0.0001) was increased in participants with pretreatment drug resistance to at least one prescribed drug, but not in those with pretreatment drug resistance and fully active ART. CD4 count increased less in participants with pretreatment drug resistance than in those without (35 cells per μL difference after 12 months; 95% CI 13–58; p=0.002).

Interpretation

At least three fully active antiretroviral drugs are needed to ensure an optimum response to first-line regimens and to prevent acquisition of drug resistance. Improved access to alternative combinations of antiretroviral drugs in sub-Saharan Africa is warranted.

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INTRODUCTION

A public-health approach based on standardised, affordable drug regimens and limited laboratory monitoring has been crucial for the scale-up of combination antiretroviral therapy (ART) in sub-Saharan African countries with high prevalences of HIV-1 [1]. By 2010, more than 4 million people with HIV-1 in sub-Saharan Africa were receiving ART—about 40% of those in need [2]. Mutations in the HIV genome that confer drug resistance can diminish the virological response to ART [3-6]. Drug-resistant variants, acquired because of selective pressure in people receiving ART, can be transmitted to people newly infected with HIV [7, 8]. In east, central, and southern Africa, reports suggest that transmitted drug-resistance has increased, in parallel with the widespread distribution of ART [9-12].

In developed countries, drug-resistance testing to guide first-line therapy choices mitigates virological failure in people who have a transmitted drug-resistant strain [3-6]. By contrast, in the absence of drug-resistance testing in resource-limited countries, standard first-line combinations of antiretroviral drugs including two nucleoside reverse transcriptase inhibitors (NRTIs) and one non-nucleoside reverse transcriptase inhibitor (NNRTI) are prescribed empirically [13, 14]. The effect of emerging pretreatment drug resistance on the virological and immunological responses to the standard first-line ART regimens in sub-Saharan Africa has not been assessed.

This study investigates the effect of pretreatment drug resistance on the virological and immunological responses and the acquisition of drug resistance after the first year of ART and additional risk factors for virological failure and acquisition of drug resistance in the PharmAccess African Studies to Evaluate Resistance Monitoring (PASER-M) cohort [15].

METHODS

Study design and participants

PASER-M is a multicentre prospective cohort of people with HIV-1 receiving ART at 13 clinics in Kenya (two clinics), Nigeria (one), South Africa (three), Uganda (three), Zambia (three), and Zimbabwe (one). Cohort characteristics [15] and pretreatment drug resistance mutations in the antiretroviral-naive cohort [10] have been described previously.

Patients were included if they were aged 18 years or older, HIV-1 infected, and started first-line ART in accordance with national guidelines—ie, when with advanced immunodeficiency (a CD4 cell count less than 200 cells per µL) or advanced HIV disease (WHO clinical stage 3 or 4) [13]. To prevent recruitment of patients who were already receiving

ART, people who had received a first-line regimen in the previous 30 days were excluded. Use of ART (more than 30 days previously), or any previous monotherapy, dual therapy, or use of antiretroviral prophylaxis was allowed. Our analysis included participants who received a standard first-line regimen containing two NRTIs and an NNRTI, excluding those who had started receiving three NRTIs only or a regimen containing a protease inhibitor. Other exclusion criteria were pregnancy at study screening and—tested in Nigeria only—HIV-2 co-infection.

Participants provided written, informed consent at enrolment. The study protocol was approved by the appropriate national and local research ethics committees at the collaborating sites and by the Academic Medical Centre, University of Amsterdam, Netherlands.

Procedures

Participants were followed up in accordance with the local standard of care guidelines. Medical staff at each site extracted routine clinical and laboratory data recorded in medical records into standard case-report forms, which were then entered into a central online database. CD4 cell counts recorded closest to the ART start date were used as pretreatment counts. Throughout the study, a data monitoring team verified source data and reviewed study data.

Drug adherence was assessed at each follow-up clinic visit by two measures of self-reported adherence. For 3 day self-reports, we counted the number of follow-up visits at which the patients reported to have missed any pills during the previous 3 days. For the 30 day visual analogue scale, we averaged the number of pills taken at all follow-up visits and classified adherence accordingly as less than 80%, 80–94%, 95–99%, or 100% [16, 17].

Patients were included in one of the following outcome categories: still on a first-line ART regimen after 12 months of follow-up, discontinued ART, transferred out, lost to follow-up, died, or switched to second-line ART because of treatment failure as judged by local criteria [13]. A single-drug substitution because of toxicity or intolerance was not classed as a regimen switch. Time was measured from the start of ART and ended at the earliest of either last follow-up visit or 12 months after starting ART (from 11 to 15 months); possible treatment interruptions were ignored.

Plasma was sampled at the baseline visit, after 12 months of therapy, and—if applicable—earlier, in case of treatment failure, and stored for later assessment of HIV RNA viral load and genotypic drug resistance. HIV viral load results after 12 months of follow-up

for participants who were still on a first-line regimen were classed as either suppression (<400 copies per mL) or failure (≥400 copies per mL).

All virological testing was done at either of two reference laboratories: one in South Africa or one in Uganda [10]. Viral loads were measured with NucliSens EasyQ real-time assay (version 2.0) or COBAS Ampliprep/COBAS Taqman assay. For baseline and follow-up specimens with a viral load greater than 1000 copies per mL, population-based genotyping of HIV protease and reverse transcriptase was done with in-house sequencing methods. HIV subtypes were inferred from the *pol* sequences with the STAR algorithm [18] and confirmed with REGA (version 2.0) [19]. Genotypic drug resistance was defined as the presence of at least one major aminoacid substitution included in the International Antiviral Society USA mutation list of December, 2010, [20] including revertant mutations at codon 215 [21]. NRTIs, NNRTIs, and protease inhibitors were included.

Pretreatment drug resistance was defined in two steps. We used the International Antiviral Society USA mutation list to distinguish between participants with a virus with at least one drug-resistance mutation and those with none [20]. For participants with a virus with at least one drug-resistant mutation, we used the Stanford drug-susceptibility algorithm (version 6.0.9) [22] to classify participants into those receiving fully active ART (Stanford levels 1 [susceptible] or 2 [potential low-level resistance] for all prescribed drugs), or those receiving partly active ART (Stanford levels 3 [low-level resistance], 4 [intermediate resistance], or 5 [high-level resistance] for at least one of the prescribed drugs). All sequences have been deposited in GenBank (for accession numbers see webappendix panel).

Statistical analysis

We estimated a required sample size of at least 190 people per site on the basis of anticipated virological failure and drop-out rates, and balanced against the cost of genotypic testing [15]. We did group comparisons for categorical data with a χ^2 test, and for continuous data with a one-way ANOVA or Kruskal-Wallis test [23], as appropriate. We used multilevel analysis with random intercepts to assess the effect of baseline and prospective factors at the level of individuals and sites (while accounting for clustering of observations within sites) on two outcomes after 12 months of therapy—virological failure and acquired drug-resistance (defined as the presence of at least one International Antiviral Society USA drug-resistance mutation and viral load >1000 copies per mL). To account for missing data due to participant attrition, we did multiple imputations with the Markov chain Monte Carlo approach with five simulation datasets.

The main analysis was a model that incorporated the following baseline characteristics: pretreatment drug resistance, age, sex, clinical stage of disease, initial NRTI backbone

(stavudine, zidovudine, tenofovir, or abacavir) and NNRTI drug (efavirenz or nevirapine), previous antiretroviral drug exposure (ART, single-dose nevirapine for prevention of mother-to-child transmission, combination prophylaxis for prevention of mother-to-child transmission, or other), pretreatment CD4 cell count (<50, 50–199, or ≥200 cells per μL), pretreatment viral load (log_{10}-transformed, fitted as a continuous variable), HIV subtype, anaemia, body mass index (fitted as a continuous variable), cost to patient, year of ART initiation, government or non-government site, years of on-site ART experience, and patient to staff ratio. The prospective model also includes CD4 cell count increase 6 months after ART initiation (fitted as a categorical variable), single-drug substitution, 3 day and 30 day adherence (fitted as categorical variables), and routine viral-load test at 6 months after ART initiation (available at seven of 13 sites). All variables were assessed univariately and those associated ($p<0.10$) with the outcome were entered stepwise into the multivariate model. Biologically plausible interactions were examined in all multivariate models. We did several sensitivity analyses (webappendix table 3).

We estimated the mean changes in CD4 cell counts between the pretreatment drug resistance groups over intervals of 3 months by use of linear mixed models. All CD4 cell counts measured routinely before and after start of ART were used. The model was adjusted for age, sex, pretreatment CD4 cell count and viral load, HIV subtype, previous antiretroviral drug exposure, year of ART initiation, types of NRTI and NNRTI, and 30 day adherence. A sensitivity analysis included only participants who had viral suppression (HIV RNA <50 copies per mL) after 12 months of ART.

Reported p values are two-sided and a p value below 0·05 was judged significant. All analyses were done with Stata (version 11).

Role of the funding source

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

RESULTS

Between March, 2007, and September, 2009, 2733 participants were recruited (median 221 per site, range 116–239; figure 1). 2588 participants (95%) reported no previous exposure to antiretroviral drugs, 145 (5%) had previously had antiretroviral drugs, including ART (n=61), monotherapy or dual therapy (four), single-dose nevirapine for prevention of mother-to-child transmission (43), combination regimen for prevention

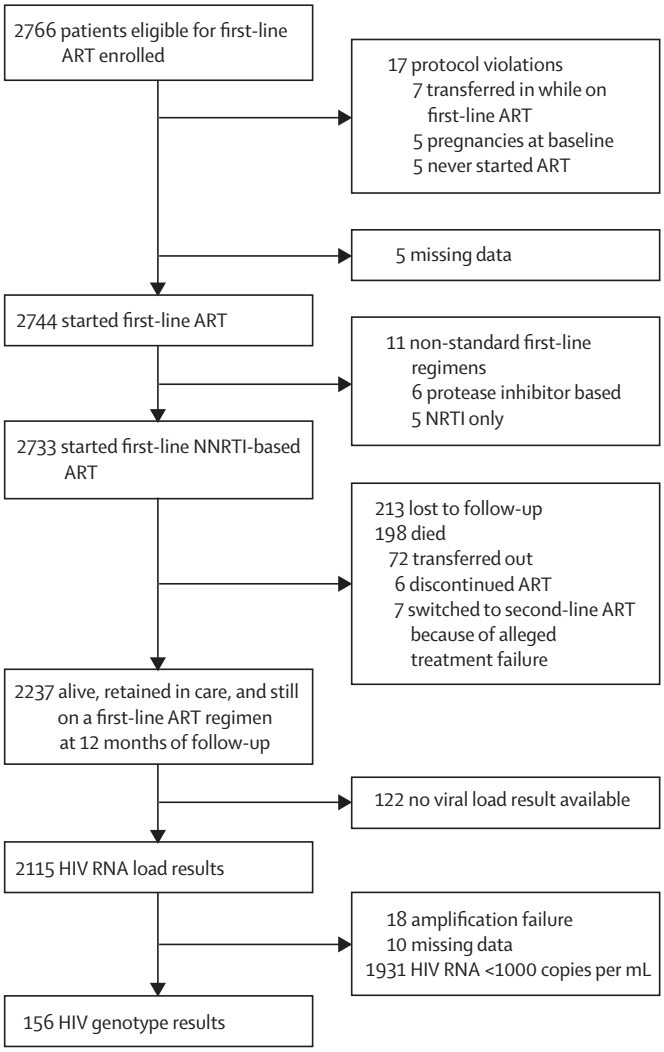


Figure 1. Study profile.
ART, antiretroviral therapy; NRTI, nucleoside reverse transcriptase inhibitor; NNRTI, non-nucleoside reverse transcriptase inhibitor

of mother-to-child transmission (19), postexposure prophylaxis (one), or unknown (23). Webappendix table 1 shows participant characteristics by site.

A pretreatment drug resistance test result was available for 2579 participants (94%; table 1). Pretreatment drug resistance was detected in 175 participants (7%), of whom 52 (30%) received fully-active ART and 123 (70%) had reduced susceptibility to at least one prescribed drug. Of these 123 participants, 99 (80%) had high-level resistance (Stanford 5), 11 (9%) had intermediate-level resistance (Stanford 4), and 13 (11%) had low-level

Table 1. Patients' baseline characteristics

	All (n=2579)	No PDR (n=2404)	PDR (n=175)	P-value
Sex				0.494
Female	1493 (57.9)	1396 (58.1)	97 (55.4)	
Male	1086 (42.1)	1008 (41.9)	78 (44.6)	
Age – mean years (SD)	37.8 (9.0)	37.8 (9.0)	37.6 (9.3)	0.7361
18-29	506 (19.6)	470 (19.6)	36 (20.6)	0.947
30-39	1166 (45.2)	1088 (45.3)	78 (44.6)	
≥40	907 (35.2)	846 (35.2)	61 (34.9)	
Country				<0.0001
Zambia	551 (21.4)	522 (21.7)	29 (16.6)	
South Africa	601 (23.3)	566 (23.5)	35 (20.0)	
Uganda	606 (23.5)	530 (22.1)	76 (43.4)	
Kenya	424 (16.5)	405 (16.9)	19 (10.9)	
Zimbabwe	204 (7.9)	194 (8.1)	10 (5.7)	
Nigeria	193 (7.5)	187 (7.8)	6 (3.4)	
Calendar year of initiation				0.014
2007	618 (24.0)	585 (24.3)	33 (18.9)	
2008	1596 (61.9)	1470 (61.2)	126 (72.0)	
2009	366 (14.2)	349 (14.5)	16 (9.1)	
WHO clinical stage at initiation				0.936
1 or 2	1000 (38.8)	930 (38.7)	70 (40.0)	
3	1150 (44.6)	1073 (44.6)	77 (44.0)	
4	429 (16.6)	401 (16.7)	28 (16.0)	
Previous antiretroviral experience				<0.0001
No	2442 (94.7)	2302 (95.8)	140 (80.0)	
Yes	115 (4.5)	82 (3.4)	33 (18.9)	
Unknown	22 (0.9)	20 (0.8)	2 (1.1)	
Type of initial NRTI-backbone *				0.773
Zidovudine based	960 (37.2)	900 (37.4)	60 (34.3)	
Stavudine based	685 (26.6)	639 (26.6)	46 (26.3)	
Tenofovir based	870 (33.7)	805 (33.5)	65 (37.1)	
Abacavir based	64 (2.5)	60 (2.5)	4 (2.3)	
Type of initial NNRTI				0.459
Efavirenz	1542 (59.8)	1442 (60.0)	100 (57.1)	
Nevirapine	1037 (40.2)	962 (40.0)	75 (42.9)	
Pre-treatment body-mass index, median kg per m^2 (IQR) †	21.0 (18.8-24.0)	21.0 (18.3-23.7)	21.0 (18.4-23.6)	0.3553
Pre-treatment hemoglobin, median gram per dL (IQR) ‡	11.4 (9.9-12.9)	11.4 (9.9-12.9)	11.8 (10.5-13.2)	0.2300

Table 1 (continued)

	All (n=2579)	No PDR (n=2404)	PDR (n=175)	P-value
Pre-treatment CD4 cell count, median cells per µL (IQR) §	133 (62-204)	133 (62-204)	129 (53.5—204.5)	0.7148
<50	534 (20.8)	492 (20.5)	42 (24.0)	0.490
50-99	432 (16.8)	408 (17.0)	24 (13.7)	
100-199	910 (35.4)	845 (35.3)	65 (37.1)	
≥200	695 (27.0)	651 (27.2)	44 (25.1)	
Pre-treatment HIV-RNA, median $\log_{10}$ copies per mL (IQR) ¶	5.00 (4.36-5.59)	5.04 (4.46-5.62)	5.16 (4.58-5.74)	0.591 ||

Data are n (%), mean (SD), or median (IQR). Data are from participants who initiated an NNRTI-based first-line regimen and had a pretreatment genotypic resistance test result (n=154 excluded). p values refer to the comparison between PDR and no PDR groups. *Combined with lamivudine or emtricitabine. †Data available for 2562 patients. ‡Data available for 2526 patients. §Data available for 2568 patients. ¶Data available for 2561 patients. ||Adjusted for assay. PDR, pretreatment drug-resistance; NRTI, nucleoside reverse transcriptase inhibitor; NNRTI, non-nucleoside reverse transcriptase inhibitor;

resistance (Stanford 3) to at least one prescribed drug. The median number of active drugs for participants with pretreatment drug resistance to at least one prescribed drug was 2·0 (IQR 1.25–2.0). 1405 participants (54%) had HIV-1 subtype C, then 638 (25%) A, 296 (11%) D, 117 (5%) A/G recombinant, 68 (3%) G, 48 (2%) other recombinants, seven (<1%) other subtypes, and five (<1%) B. The distribution of pretreatment drug resistance and previous exposure to antiretroviral drugs did not differ between the initial ART regimens (data not shown). The prevalences of all pretreatment drug-resistance mutations are listed in webappendix table 2.

Total follow-up was 2358 person-years, with a median of 11·9 months (IQR 11.2–12.2). Of 2733 participants, 2237 (82%) were retained in care and still receiving first-line ART after 12 months of follow-up. 489 (18%) were not retained, 198 (7%) because they had died, 213 (8%) lost to follow-up, 72 (3%) transferred out, and six (<1%) discontinued ART. Seven (<1%) patients switched to a second-line regimen in the first year of ART because of treatment failure (figure 1), one of whom had pretreatment drug resistance (webappendix figure 1). A slightly smaller proportion of patients with pretreatment drug resistance were retained on first-line ART (133 [76%]) than were those without (1979 [82%]; p=0·036); whereas all-cause mortality did not differ significantly between the groups (17 [10%] *vs* 171 [7%]; p=0·201).

1942 participants (87%) were still taking their original regimen after 12 months, and 295 (13%) had a single-drug substitution because of toxicity, intolerance, or other reasons. 12 patients (1%) interrupted ART because of severe intolerance. 2218 participants (81%) had not missed any pills in the 3 days before any follow-up visit. 1517 patients (56%)

had 100% 30 day adherence, 726 (27%) had 95–99% adherence, 201 (7%) had 80–94% adherence, and 37 (1%) had <80% adherence. Pretreatment drug resistance and adherence were not associated (data not shown).

Viral load at 12 months' follow-up was assessed for 2115 (95%) of 2237 participants still taking first-line ART. 213 of 2733 patients (70%, 95% CI 68–71) who started first-line ART had viral suppression; 213 of 2115 (90%, 89–91) of those who were still on first-line ART had viral suppression (figure 1). Virological suppression at 12 months did not differ significantly between participants without pretreatment drug resistance (1697 [91%]) and those with pretreatment drug resistance and fully active ART (36 [86%]; p=0.274), but was significantly lower in participants with resistance to at least one prescribed drug (63 [75%]; p<0·0001).

Of the 184 participants with a viral load greater than 1000 copies per mL at 12 month follow-up, 156 (85%) specimens were successfully genotyped: 113 (72%) had one or more major drug-resistance mutation. Drug-resistance mutations were associated with NRTIs (94, 60%), NNRTIs (98, 63%) and protease inhibitors (two, 1%). Dual-class resistance to NRTIs and NNRTIs was detected in 80 participants (51%), and no triple-class resistance was detected. Of the 113 participants who had one or more major drug-resistance mutation after 12 months of ART, 20 (18%) had pretreatment drug resistance, 87 (77%) did not, and status was unknown in six (5%).

Compared with participants without pretreatment drug resistance, people who had resistance to at least one prescribed drug had an increased risk of virological failure (odds ratio [OR] 2.13, 95% CI 1.44–3.14; p<0·0001; table 2) and acquired drug-resistance (2.30, 1.55–3.40; p<0.0001; table 3). By contrast, for those who had pretreatment drug resistance and were prescribed a fully active regimen, the ORs for virological failure (1.01, 0.55–1.87; p=0.964; table 2) or acquired drug-resistance (0.97, 0.51–1.58; p=0.934; table 3) were not increased, compared with those without pretreatment drug resistance (webappendix table 3). Additionally, compared with participants with pretreatment drug resistance and reduced susceptibility to at least one prescribed drug, the risks of virological failure (0.48, 0.23–0.97, p=0.041) and acquired drug-resistance (0.42, 0.20–0.88; p=0.022) were reduced in those with pretreatment drug resistance and fully active ART.

Independent of pretreatment drug resistance, participants who had virological failure or acquired drug resistance were more likely to have been previously exposed to ART or prevention of mother-to-child transmission, to have high pretreatment viral load, and to be younger (for women) at ART initiation, than were participants who did not (tables 2, 3). Participants who acquired drug resistance were also more likely to have a

Table 2. Risk factors of virological failure in the first year of first-line ART

	Number of events	Univariate			Multivariate					
					Baseline model			Prospective model		
		OR	95%CI	P	OR	95%CI	P	OR	95%CI	P
Total	**213**									
Pre-treatment drug-resistance										
No PDR	174	1			1			1		
PDR and fully-active ART	6	1.62	0.67, 3.92	0.282	1.01	0.55, 1.87	0.964	1.07	0.54, 2.11	0.853
PDR and partially-active ART	21	3.31	1.96, 5.59	<0.001	2.13	1.44, 3.14	<0.001	2.21	1.40, 3.50	0.001
Sex										
Women	108	1			1			1		
Men	105	1.53	1.15, 2.03	0.003	0.86	0.39, 1.86	0.695	0.52	0.21, 1.30	0.162
Age (yrs), mean (sd) *		0.76	0.64, 0.91	0.002						
For men					0.87	0.76, 1.01	0.06	0.87	0.61, 1.04	0.114
For women					0.74	0.64, 0.85	<0.001	0.67	0.74, 1.03	<0.001
Calendar year of ART initiation										
2007	64	1			1			1		
2008	116	0.55	0.36, 0.84	0.006	0.87	0.66, 1.13	0.291	0.72	0.53, 0.99	0.041
2009	33	0.60	0.33, 1.11	0.104	0.69	0.46, 1.04	0.075	0.67	0.42, 1.07	0.092
WHO clinical stage										
1 or 2	76	1								
3 or 4	137	1.34	1.00, 1.80	0.054						
Body Mass Index, kg/m^2 †		0.81	0.68, 0.97	0.019						
Pre-treatment viral load (log_{10} c/mL), median (IQR)		1.29	1.10, 1.52	0.002	1.20	1.08, 1.34	0.001	1.13	0.99, 1.28	0.071
Previous antiretroviral experience										
None	194	1			1			1		
ART	10	3.64	1.73, 7.69	0.001	2.62	1.50, 4.57	0.001	3.10	1.65, 8.81	<0.001
Single-dose nevirapine for PMTCT	5	1.95	0.73, 5.20	0.181	1.66	0.84, 3.30	0.148	2.04	0.94, 4.47	0.073
Other	3	2.01	0.57, 7.10	0.278	1.91	0.84, 4.36	0.124	1.86	0.75, 4.60	0.182
Type of initial NRTI-backbone ‡										
Zidovudine based	102	1								
Stavudine based	43	0.63	0.43, 0.91	0.015						
Tenofovir based	63	0.72	0.52, 1.01	0.057						
Abacavir based	5	1.04	0.40, 2.71	0.942						
Type of initial NNRTI §										
Efavirenz	104	1			1			1		
Nevirapine	109	1.50	1.13, 1.99	0.005	0.98 §	0.80, 1.20	0.864	1.12	0.88, 1.41	0.363

Table 2 (continued)

	Number of events	Univariate			Multivariate					
					Baseline model			Prospective model		
		OR	95%CI	P	OR	95%CI	P	OR	95%CI	P
CD4 gain six months after initiation, cells per µL ¶										
<0 (decrease)	18	1						1		
0-99	41	0.41	0.22, 0.76	0.004				0.36	0.22, 0.60	<0.001
100-199	28	0.30	0.16, 0.57	<0.001				0.27	0.16, 0.45	<0.001
≥200	17	0.27	0.13, 0.55	<0.001				0.31	0.18, 0.54	<0.001
3-day self-report adherence, no. ¶										
0	126	1						1		
1	52	2.39	1.76, 3.42	<0.001				1.31	0.94, 1.83	0.114
2	27	6.41	3.83, 10.73	<0.001				1.67	0.96, 2.88	0.068
3 or more	8	7.92	3.16, 19.83	<0.001				1.87	0.71, 4.94	0.208
30-day VAS adherence, %¶										
100	99	1						1		
95-99	57	1.10	0.76, 1.57	0.623				0.63	0.47, 0.85	0.003
94-80	38	3.69	2.39, 5.70	<0.001				1.75	1.14, 2.69	0.010
<80	10	9.44	3.97, 22.45	<0.001				3.70	1.71, 8.04	0.001

Table shows results of multilevel logistic regression, including the data from all participants who initiated a NNRTI-based first-line regimen. 3 day self-report is defined as the number of follow-up clinic visits at which the patient reported to have missed any pills in the 3 previous days. 30 day VAS is defined as the mean visual analogue score at all follow-up clinic visits. Total number of events=213. *Odds ratio for 10-year increase in age. †Odds ratio for 5 kg/m2 increase. ‡Combined with lamivudine or emtricitabine. §Inverse-probability weighting for NNRTI assignment: OR 0.91 (95% CI 0.69–1.19; p=0.475). ¶The baseline model did not include data for prospectively collected parameters. ART, antiretroviral therapy; NRTI, nucleoside reverse transcriptase inhibitor; NNRTI, non-nucleoside reverse transcriptase inhibitor; PMTCT, prevention of mother-to-child transmission; VAS, visual analogue score; OR, odds ratio; PDR, pretreatment drug resistance.

pretreatment CD4 count less than 50 cells per µL than were those who did not. Of the measures obtained prospectively after ART initiation, no CD4 cell count increase during the first 6 months of ART, and suboptimum (<95%) 30 day adherence were associated with virological failure (table 2) and acquired drug-resistance (table 3) by month 12. In the univariate analysis the following covariables were not associated with virological failure: pretreatment CD4 cell count, treatment cost, severe anaemia at initiation, HIV subtype, site administration, site ART experience, patient to staff ratio, single-drug substitution, viral-load test at 6 months after initiation. In univariate analysis the following covariables were not associated with acquired drug resistance: free treatment, severe anaemia at initiation, body-mass index, HIV-1 subtype, site administration, site ART experience, patient to staff ratio, single-drug substitution, viral-load test at 6 months after initiation. No significant effect modification across strata of pretreatment drug resistance and covariables occurred.

Table 3. Risk factors of acquired drug-resistance in the first year of first-line ART

	Number of events	Univariate			Multivariate					
					Baseline model			Prospective model		
		OR	95%CI	P	OR	95%CI	P	OR	95%CI	P
Total	**113**									
Pre-treatment drug-resistance										
No PDR	87	1			1			1		
PDR and fully-active ART	4	2.11	0.73, 6.11	0.166	0.97	0.51, 1.58	0.934	1.05	0.52, 2.15	0.884
PDR and partially-active ART	16	5.03	2.77, 9.12	<0.001	2.30	1.55, 3.40	<0.001	2.42	1.53, 3.83	<0.001
Sex										
Women	56	1			1			1		
Men	57	1.54	1.05, 2.26	0.026	0.78	0.35, 1.76	0.555	0.45	0.17, 1.17	0.102
Age (yrs), mean (sd) *		0.75	0.59, 0.94	0.014						
For men					0.90	0.77, 1.04	0.151	0.91	0.76, 1.08	0.280
For women					0.75	0.65, 0.87	<0.001	0.67	0.56, 0.80	<0.001
Calendar year of ART initiation										
2007	33	1			1			1		
2008	62	0.56	0.32, 0.98	0.042	0.97	0.74, 1.27	0.819	0.78	0.57, 1.08	0.132
2009	18	0.63	0.29, 1.41	0.263	0.83	0.55, 1.27	0.394	0.76	0.47, 1.22	0.255
WHO clinical stage										
1 or 2	38	1								
3 or 4	75	1.46	0.97, 2.18	0.068						
Pre-treatment CD4 count, cells per µL										
<50	50	1			1			1		
50-199	43	0.62	0.40, 0.97	0.037	0.67	0.55, 0.83	<0.001	0.64	0.50, 0.81	<0.001
≥200	20	0.35	0.20, 0.63	<0.001	0.60	0.47, 0.76	<0.001	0.58	0.44, 0.77	<0.001
Pre-treatment viral load (log_{10} c/mL), median (IQR)		1.43	1.13, 1.80	0.002	1.12	1.00, 1.26	0.048	1.04	0.91, 1.19	0.594
Previous antiretroviral experience										
None	100	1			1			1		
ART	8	5.83	2.56, 13.31	<0.001	2.76	1.58, 4.85	<0.001	3.22	1.71, 6.05	<0.001
Single-dose nevirapine for PMTCT	3	2.16	0.63, 7.45	0.222	1.99	0.99, 3.99	0.052	2.34	1.06, 5.18	0.035
Other	1	2.21	0.16, 9.40	0.855	2.11	0.90, 4.93	0.084	1.98	0.78, 4.99	0.150
Type of initial NRTI-backbone †										
Zidovudine based	58	1			1			1		
Stavudine based	12	0.31	0.16, 0.58	<0.001	1.13	0.85, 1.50	0.398	1.05	0.76, 1.44	0.759
Tenofovir based	39	0.79	0.52, 1.20	0.272	1.08	0.84, 1.39	0.557	1.02	0.76, 1.38	0.883
Abacavir based	4	1.47	0.51, 4.28	0.478	1.99	1.13, 3.51	0.018	1.53	0.78, 3.00	0.221

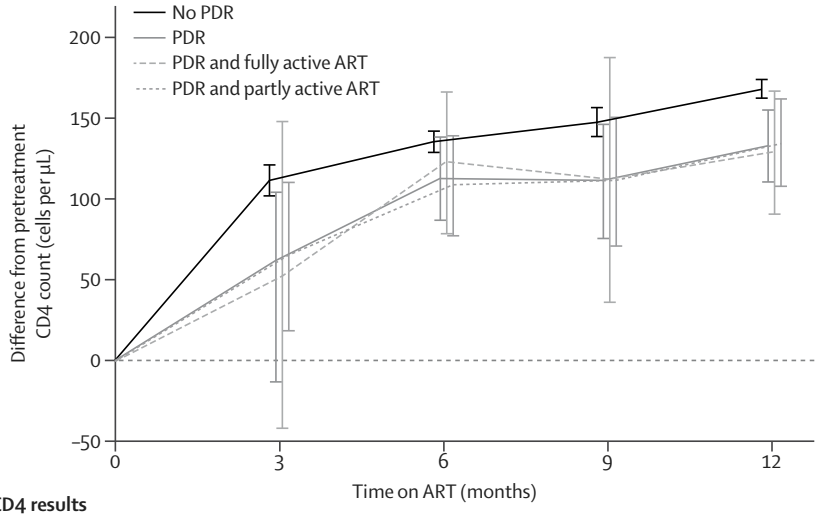
Table 3 (continued)

	Number of events	Univariate			Multivariate					
					Baseline model			Prospective model		
		OR	95%CI	P	OR	95%CI	P	OR	95%CI	P
Type of initial NNRTI										
Efavirenz	52	1			1			1		
Nevirapine	61	1.65	1.11, 2.46	0.014	0.96‡	0.77, 1.20	0.735	1.06	0.82, 1.38	0.636
CD4 gain six months after initiation, cells per µL §										
<0 (decrease)	11	1						1		
0-99	32	0.56	0.27, 1.16	0.117				0.34	0.20, 0.57	<0.001
100-199	13	0.24	0.10, 0.55	0.001				0.22	0.13, 0.38	<0.001
≥200	7	0.19	0.071, 0.51	0.001				0.27	0.15, 0.48	<0.001
3-day self-report adherence, no. §										
0	70	1						1		
1	25	1.87	1.16, 3.02	0.010				0.79	0.56, 1.13	0.205
2	12	3.90	2.00, 7.61	<0.001				0.64	0.35, 1.17	0.147
3 or more	6	8.44	3.15, 22.62	<0.001				1.03	0.37, 2.90	0.952
30-day VAS adherence, % §										
100	48	1						1		
95-99	35	1.50	0.93, 2.42	0.097				0.86	0.63, 1.16	0.325
94-80	24	4.46	2.60, 7.66	<0.001				2.46	1.57, 3.84	<0.001
<80	5	7.99	2.76, 23.11	<0.001				4.97	2.30, 10.74	<0.001

Table shows results of multilevel logistic regression, including the data from all participants who initiated a NNRTI-based first-line regimen. *OR for 10-year increase in age. †Combined with lamivudine or emtricitabine. ‡Inverse-probability weighting for NNRTI-assignment: OR 0.78 (95% CI 0.59–1.04; p=0.088). §The baseline model did not include data for prospectively collected parameters. PDR, pretreatment drug resistance; ART, antiretroviral therapy; NRTI, nucleoside reverse transcriptase inhibitor; NNRTI, non-nucleoside reverse transcriptase inhibitor; PMTCT, prevention of mother-to-child transmission; VAS, visual analogue score; OR, odds ratio.

The median gain in CD4 count between the start of ART and 6 months' follow-up was 110 (IQR 57–186) cells per µL and 149 (IQR 76–239) cells per µL at 12 months. Compared with participants without pretreatment drug resistance, CD4 count increased less in the first 3 months of ART (49 cells per µL; 95% CI 7–90; p=0.021) up to 12 months (35 cells per µL; 13–58; p=0.002) in all participants with pretreatment drug resistance; the difference was 40 cells per µL per 12 months (2–78; p=0.041) for those with pretreatment drug resistance and fully active ART, and 33 cells per µL (6–61; p=0.015) for those with resistance to at least one prescribed drug (figure 2).

In a sensitivity analysis, which only included participants who had viral suppression (viral load <50 copies per mL) at 12 months' follow-up, the reduced CD4 increase for all participants with pretreatment drug resistance was of borderline statistical significance



Number of CD4 results					
No PDR	2396	442	1095	489	1527
PDR	175	24	80	38	113
PDR and fully active ART	52	4	26	10	39
PDR and partly active ART	123	20	54	28	72

Figure 2. Change in CD4 cell count, by pretreatment drug resistance.
Linear mixed model (n=2439) adjusted for age, sex, pre-treatment CD4 count, pretreatment HIV RNA load, HIV-1 subtype, year of ART initiation, NRTI-backbone, NNRTI-drug, previous antiretroviral drug exposure, and adherence; not adjusted for HIV RNA loads at 12 months of therapy. Vertical bars = 95% CI. PDR, pre-treatment drug resistance; ART, antiretroviral therapy.

(28 cells per µL; 95% CI 1–55; p=0.039), compared with those without pretreatment drug resistance (webappendix figure 2).

DISCUSSION

Pretreatment drug resistance was strongly associated with virological failure and development of drug-resistance after the first year of standard first-line NNRTI-based ART in patients in sub-Saharan Africa who received partly active regimens—ie, that included at least one drug to which the virus had reduced susceptibility. By contrast, the response to ART was not reduced in patients who had a drug-resistant virus when the prescribed regimen was predicted to be fully active. Our findings seem to be robust in sensitivity analyses that adjusted for possible effects of previous antiretroviral drug exposure and the M184V mutation (webappendix table 3). These findings are largely in agreement with results from studies in developed countries (panel) [3-6]. Notably, 70% of participants who had pretreatment drug resistance were empirically started on a suboptimum first-line regimen—nearly 5% of the total study population. In view of the limited potency and low genetic barrier of NNRTI-based regimens, our study emphasises

the need for at least three fully active antiretroviral drugs in first-line regimens in Africa to ensure an optimum virological response and to prevent the acquisition of NRTI and NNRTI drug-resistance mutations.

Overall, CD4 cell count recovered well during the first year of ART in patients with pretreatment drug resistance, although it was reduced compared with those without resistance. This difference is probably due to the higher proportion with virological failure among patients with pretreatment drug resistance, rather than by a direct effect of drug-resistant virus on CD4 cell count. The small difference in CD4 gain after adjustment for differences in viral load at 12 months of ART, could be due to the longer time needed to achieve viral suppression or by residual viral replication below the detection level of the HIV RNA assay in the pretreatment drug resistance group, compared with the group without. However, the clinical relevance of this difference might be small, because the overall immunological response in participants with pretreatment drug resistance was favourable. By contrast with a European study (panel) [5], which showed a reduced CD4 cell count increase only in patients with transmitted resistance to at least one prescribed drug, but not in patients with transmitted resistance who received a fully active regimen, immunological outcomes did not differ between the ART susceptibility subgroups in our study, which might be because of a lack of statistical power.

Panel: Research in context

Systematic review

We searched PubMed for English-language studies, published between January, 2006, and September, 2011, with a sample size greater than 100, that assessed the effect of pretreatment HIV-1 drug resistance on response to first-line antiretroviral therapy. We identified seven cohort studies from Europe and none from resource-limited countries (appendix pp 9–10). Some studies showed no significant association between the presence of pretreatment drug resistance and virological [24-26] or immunological [24, 25] response to antiretroviral therapy. Other studies reported a decreased virological response in patients with pretreatment drug resistance compared with those without [3, 6]. A collaborative study including 10 056 patients from 25 European cohorts showed an increased risk of virological failure in patients with pretreatment drug resistance to at least one prescribed drug compared with those without pretreatment drug resistance, but no increased risk for those with pretreatment drug resistance who received fully active ART [5].

Interpretation

Our study confirms findings from studies done in high-income countries. The risk of virological failure and acquired drug resistance for patients in sub-Saharan Africa receiving non-nucleoside reverse transcriptase inhibitor-based regimens with pretreatment drug resistance to at least one prescribed drug was increased compared with those without pretreatment drug resistance and those with pretreatment drug resistance who received a fully active regimen. Improved access to alternative drugs with different modes of action and without cross-resistance to nucleoside reverse transcriptase inhibitors and non-nucleoside reverse transcriptase inhibitors is warranted in resource-limited countries to ensure continued effectiveness of HIV/AIDS treatment.

Independent of pretreatment drug resistance, previous use of ART was strongly associated with virological failure and acquired drug-resistance. Previous exposure to ART might result in minority-resistant viral strains not detected by population-based sequencing. Minority NNRTI resistance mutations might contribute to virological failure in patients starting an NNRTI-based regimen [27-32]. Furthermore, women who had had single-dose nevirapine to prevent perinatal transmission of HIV-1 had a slightly higher risk of virological failure than did antiretroviral-naive women. A randomised study [33] in Botswana showed that previous exposure to peripartum single-dose nevirapine results in increased rates of virological failure if given less than 6 months before the start of subsequent NNRTI-based ART. For this reason, the revised 2010 WHO HIV treatment guidelines [14] recommend initiating a regimen based on a protease inhibitor in women with previous exposure to antiretrovirals for prevention of mother-to-child transmission if they have received single-dose nevirapine alone or in combination with other drugs without an NRTI tail within 12 months of starting long-term ART, irrespective of the presence of NNRTI-associated pretreatment drug resistance. Because of the small numbers of women who had received ART for prevention of mother-to-child transmission in our study, we could not differentiate according to the time since prevention of mother-to-child transmission treatment exposure.

In the absence of routine virological monitoring, clinical proxy measures of treatment failure, obtained prospectively after ART initiation, might be helpful for clinical decision making as an early warning signal. In our study, we noted that failure to achieve an increase in CD4 cell count by month 6 as well as prolonged non-adherence below 95% was associated with virological failure and the acquisition of drug resistance by month 12. Sustained suboptimum drug concentrations leads to ongoing viral replication and selection for drug-resistant variants. Suboptimum ART adherence predicts virological failure [34], the development of drug resistance [35, 36] and death [37]. Our finding supports current recommendations for NNRTI-based regimens to strive for adherence levels above 95%. Average adherence was high in our cohort, which suggests that adequate rates of adherence can be achieved in African ART programmes; although self-reported adherence measures as used in our study can be affected by recall or social desirability bias [38].

This study has some limitations. Attrition due to mortality and loss to follow-up in the first year of ART was high 489 (18% of patients enrolled), which is consistent with attrition estimates for ART programmes in sub-Saharan Africa [2, 39]. To account for the uncertainty introduced by these missing data, we used multiple imputations, which reduce bias and improve statistical efficiency. A sensitivity analysis that classed missing data as failures also suggests that our findings are robust. The overall virological failure after

12 months of therapy was 30% for all patients who started ART and 10% for those who were still on first-line ART, which compares favourably to a systematic review of 89 ART programmes in Africa [40]. Although the PASER network includes mostly free-access, routine ART programmes, non-government and urban sites were over-represented, and public and rural sites were under-represented. Therefore, caution is warranted when extrapolating the results to other settings where resource constraints might be even more substantial. Population-based sequencing cannot detect minority-resistant viral strains that are present below 25% of the viral population. Therefore, a proportion of participants with pretreatment drug resistance could have been misclassified as having none (false-negative), which might have led to underestimates of the effect on treatment outcome. Furthermore, the absence of serial blood sampling after ART initiation precluded a time-to-event analysis for virological failure and the development of pretreatment drug resistance.

Our study emphasises the need for at least three fully active antiretroviral drugs to ensure an optimum response to first-line NNRTI-based ART regimens in sub-Saharan Africa and prevent the acquisition of HIV drug resistance. The findings have important implications for public health policy. Early mortality and attrition are major challenges to the success of ART programmes in Africa. Emerging drug-resistance also jeopardises the effectiveness of standard regimens. To maintain effective HIV/AIDS treatment in Africa, further studies and mathematical modelling are needed to establish optimum strategies for the prevention of drug-resistance, ART monitoring, retention of patients, and use of protease-inhibitor-based regimens for specific high-risk groups, taking into account that pretreatment drug resistance frequencies in Africa will likely increase in the coming years [9-12]. Extended genotypic drug-resistance testing as part of population-based surveillance is needed in regions where ART is scaled up. Improved access to alternative drugs with different modes of action and without cross-resistance to NRTIs and NNRTIs is warranted in resource-limited countries to ensure the continued effectiveness of HIV/AIDS treatment.

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Contributors

TFRdW was the principal investigator. RLH, RS, CLW, WS, MvV, and TFRdW designed the study and developed the protocol. RLH, KCES, and MvV set up the study and trained and supervised study workers. CK, MS, FC, MB, KM, MW, and AO established the cohort and supervised data collection. CLW, CK, and WS supervised the laboratory testing. RLH conceived and undertook the data analyses, and drafted the manuscript. FWW checked and supervised the statistical analyses. RS, KCES, CLW, FWW, and TFRdW helped interpret the data and reviewed the manuscript. All authors reviewed and approved the final version of the manuscript.

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WEBAPPENDIX

Panel. GenBank accession numbers

All HIV-1 *pol* sequences in this study have been deposited in GenBank under the following accession numbers:
HM119603–HM120150, HQ993572- HQ995497, JN630892-JN631033

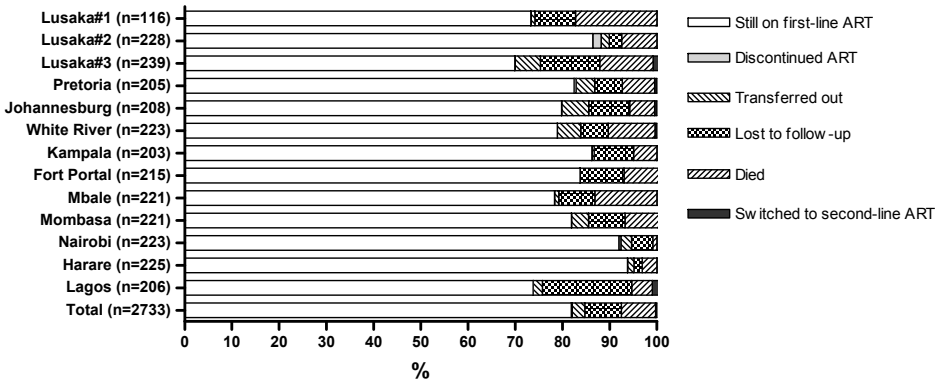

Figure 1. Bar chart of participants' clinical outcomes at 12 months of follow-up for each site.
Bart chart shows data from all participants who initiated a NNRTI-based first-line regimen.

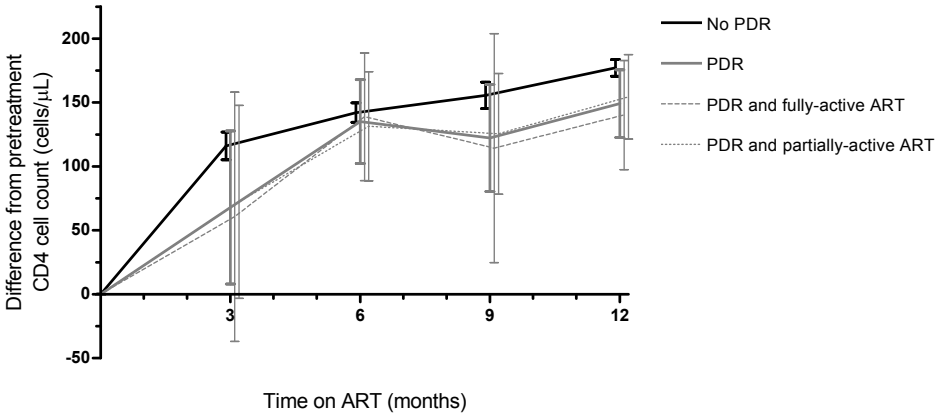

Figure 2. Change in CD4 cell count, by pre-treatment drug-resistance.
Sensitivity analysis: only includes participants with HIV-RNA<50c/mL after 12 months of therapy (n=1560). Linear mixed model adjusted for age, sex, pre-treatment CD4 count, pretreatment HIV-RNA load, HIV-1 subtype, calendar year of initiation, NRTI-backbone, NNRTI-drug, prior antiretroviral drug exposure, and adherence. Vertical bars = 95% confidence interval. PDR, pre-treatment drug-resistance; ART, antiretroviral therapy.

Table 1. Baseline characteristics of all participants, by site.

Country		Zambia			South Africa			Uganda			Kenya		Zimbabwe	Nigeria
Site	**Total (n=2733)**	**Lusaka #1 (n=116)**	**Lusaka #2 (n=228)**	**Lusaka #3 (n=239)**	**Pretoria (n=205)**	**Johannes-burg (n=208)**	**White River (n=223)**	**Kampala (n=203)**	**Fort Portal (n=215)**	**Mbale (n=221)**	**Mombasa (n=221)**	**Nairobi (n=223)**	**Harare (n=225)**	**Lagos (n=206)**
Sex														
Female	1592 (58.3)	53 (45.7)	133 (55.7)	133 (58.3)	113 (55.1)	155 (74.5)	132 (59.2)	113 (55.7)	120 (55.8)	117 (52.9)	131 (59.3)	121 (54.3)	147 (65.3)	124 (60.2)
Male	1141 (41.8)	63 (54.3)	95 (41.7)	106 (44.4)	92 (44.9)	53 (25.5)	91 (40.8)	90 (44.3)	95 (44.2)	104 (47.1)	90 (40.7)	102 (45.7)	78 (34.7)	82 (39.8)
Age – mean yrs (SD)	37.8 (9.0)	39.1 (8.9)	37.5 (8.7)	37.6 (9.4)	38.2 (8.3)	36.5 (7.4)	39.0 (10.0)	36.1 (8.8)	37.3 (10.3)	38.6 (9.6)	37.5 (8.6)	38.7 (8.7)	38.7 (8.7)	37.0 (8.2)
Calendar year of initiation														
2007	650 (23.8)	48 (41.4)	134 (58.8)	238 (99.6)	117 (57.1)	20 (9.6)	31 (13.9)	0 (0)	0 (0)	0 (0)	62 (28.1)	0 (0)	0 (0)	0 (0)
2008	1690 (61.8)	68 (58.6)	94 (41.2)	1 (0.4)	88 (42.9)	188 (90.4)	192 (86.1)	203 (100.0)	215 (100.0)	221 (100.0)	159 (72.0)	132 (59.2)	60 (26.7)	69 (33.5)
2009	393 (14.4)	0 (0)	0 (0)	0 (0)	0 (0)	0 (0)	0 (0)	0 (0)	0 (0)	0 (0)	0 (0)	91 (40.8)	165 (73.3)	137 (66.5)
WHO clinical stage at initiation														
1 or 2	1078 (39.4)	74 (63.8)	92 (40.4)	97 (40.6)	84 (41.0)	136 (65.4)	59 (26.5)	76 (37.4)	77 (35.8)	46 (20.8)	81 (36.7)	66 (29.6)	102 (45.3)	88 (42.7)
3	1205 (44.1)	19 (16.4)	115 (50.4)	126 (52.7)	71 (34.6)	53 (25.5)	96 (43.1)	78 (38.4)	90 (41.9)	139 (62.9)	124 (56.1)	106 (47.5)	102 (45.3)	86 (41.8)
4	450 (16.5)	23 (19.8)	21 (9.2)	16 (6.7)	50 (24.4)	19 (9.1)	68 (30.5)	49 (24.1)	48 (22.3)	36 (16.3)	16 (7.2)	51 (22.9)	21 (9.3)	32 (15.5)
Type of initial NRTI-backbone [a]														
Zidovudine based	1017 (37.2)	13 (11.2)	60 (26.3)	10 (4.2)	20 (9.8)	10 (4.8)	18 (8.1)	122 (60.1)	66 (30.7)	130 (58.8)	171 (77.4)	106 (47.5)	159 (70.7)	132 (64.1)
Stavudine based	728 (26.6)	13 (11.2)	10 (4.4)	86 (36.0)	2 (1.0)	193 (92.8)	204 (91.5)	0	1 (0.5)	0	47 (21.3)	107 (48.0)	65 (28.9)	0 (0)
Tenofovir based	919 (33.6)	90 (77.6)	158 (69.3)	92 (38.5)	183 (89.3)	5 (2.4)	1 (0.5)	81 (39.9)	147 (68.4)	91 (41.2)	3 (1.4)	10 (4.5)	1 (0.4)	57 (27.7)
Abacavir based	69 (2.5)	0 (0)	0 (0)	51 (21.3)	0 (0)	0 (0)	0 (0)	0 (0)	1 (0.5)	0 (0)	0 (0)	0 (0)	0 (0)	17 (8.3)
Type of initial NNRTI														
Efavirenz	1634 (59.8)	91 (78.5)	129 (56.6)	161 (67.4)	187 (91.2)	194 (93.3)	213 (95.5)	82 (40.4)	185 (86.1)	129 (58.4)	85 (38.5)	112 (50.2)	30 (13.3)	36 (17.5)
Nevirapine	1099 (40.2)	25 (21.6)	99 (43.4)	78 (32.6)	18 (8.8)	14 (6.7)	10 (4.5)	121 (59.6)	30 (14.0)	92 (41.6)	136 (61.5)	111 (49.8)	195 (86.7)	170 (82.5)

Table 1 (continued)

Country		Zambia			South Africa			Uganda			Kenya		Zimbabwe	Nigeria
Site	**Total (n=2733)**	**Lusaka #1 (n=116)**	**Lusaka #2 (n=228)**	**Lusaka #3 (n=239)**	**Pretoria (n=205)**	**Johannes-burg (n=208)**	**White River (n=223)**	**Kampala (n=203)**	**Fort Portal (n=215)**	**Mbale (n=221)**	**Mombasa (n=221)**	**Nairobi (n=223)**	**Harare (n=225)**	**Lagos (n=206)**
CD4 cell count at initiation -Median cells/μL (IQR) [b]	135 (63-206)	115.5 (47-184)	140.5 (83.5-197.5)	130 (58.5-201.5)	148 (60.5-235.5)	95.5 (28.3-163)	94.5 (34-155)	136 (53-219)	179 (99-259)	114 (50.5-177.5)	128 (61-195)	164 (104.5-223.5)	191 (114-268)	133 (66.5-199.5)
HIV RNA at initiation - median $\log_{10}$ c/ml (IQR) [c]	5.00 (4.36-5.59)	4.88 (4.09-5.67)	4.99 (4.42-5.57)	5.20 (4.73-5.68)	4.56 (3.94-5.17)	4.71 (4.10-5.32)	4.99 (4.50-5.48)	5.40 (4.96-5.84)	5.23 (4.79-5.66)	5.59 (5.03-6.14)	4.62 (3.96-5.28)	4.66 (4.07-5.25)	4.68 (4.06-5.29)	5.10 (4.53-5.66)
Pre-treatment drug-resistance [d]														
No PDR	2404 (88.0)	103 (88.8)	207 (90.8)	212 (88.7)	189 (92.2)	181 (87.0)	196 (87.9)	170 (83.7)	175 (81.4)	185 (83.7)	203 (91.9)	202 (90.6)	194 (86.2)	187 (90.8)
PDR and fully-active ART	52 (1.9)	1 (0.9)	1 (0.44)	5 (2.1)	0 (0)	3 (1.4)	7 (3.1)	12 (5.9)	9 (4.2)	3 (1.4)	5 (2.3)	2 (0.9)	4 (1.8)	0 (0)
PDR and partially-active ART	123 (4.5)	5 (4.3)	9 (4.0)	8 (3.4)	8 (3.9)	6 (2.9)	11 (4.9)	12 (5.9)	18 (8.4)	22 (10.0)	6 (2.7)	6 (2.7)	6 (2.7)	6 (2.9)
PDR unknown	154 (5.6)	7 (6.0)	11 (4.8)	14 (5.9)	8 (3.9)	18 (8.7)	9 (4.0)	9 (4.4)	13 (6.1)	11 (5.0)	7 (3.2)	13 (5.8)	21 (9.3)	13 (6.3)
Previous antiretroviral experience	122 (4.5)													
No	2588 (94.7)	110 (94.8)	213 (93.4)	232 (97.1)	182 (88.8)	197 (94.7)	214 (96.0)	188 (92.6)	197 (91.6)	217 (98.2)	211 (95.5)	214 (96.0)	211 (93.8)	202 (98.1)
Yes	122 (4.5)	4 (3.5)	11 (4.8)	5 (2.1)	21 (10.2)	11 (5.3)	5 (2.2)	15 (7.4)	17 (7.9)	2 (0.9)	9 (4.1)	6 (2.7)	14 (6.2)	2 (1.0)
Unknown	23 (0.8)	2 (1.7)	4 (1.8)	2 (0.8)	2 (1.0)	0 (0)	4 (1.8)	0 (0)	1 (0.5)	2 (0.9)	1 (0.5)	3 (1.4)	0 (0)	2 (1.0)

Data are number (%) unless stated otherwise. [a] Combined with lamivudine or emtricitabine. [b] All n =2722; [c] All n =2706; [d] At least one drug-resistance mutation of the IAS-USA list, includes 52 participants who received a fully-active regimen (Stanford levels 1 or 2) and 123 who harbored drug-resistance to at least one drug in the prescribed regimen (Stanford levels 3, 4, or 5). PDR, pre-treatment drug-resistance; NRTI, nucleoside reverse transcriptase inhibitor; NNRTI, nonnucleoside reverse transcriptase inhibitor; SD, standard deviation; IQR, interquartile range; WHO, World Health Organization.

Table 2. Pre-treatment prevalence of major IAS-USA drug-resistance mutations (n=2579).

NRTI mutations		NNRTI mutations		PI mutations	
Mutation	**N (%)**	**Mutation**	**N (%)**	**Mutation**	**N (%)**
M41L	19 (0.74)	L100I	4 (0.16)	D30N	1 (0.04)
A62V	3 (0.12)	K101P	0	V32I	0
K65R	4 (0.16)	K103N	57 (2.21)	M46I	5 (0.19)
D67N	3 (0.12)	V106A	0	M46L	6 (0.23)
T69INS	0	V106M	3 (0.12)	I47A	0
K70E	3 (0.12)	V108I	15 (0.58)	I47V	0
K70R	13 (0.50)	Y181C	25 (0.97)	G48V	0
L74V	1 (0.04)	Y181I	1 (0.04)	I50L	1 (0.04)
V75I	7 (0.27)	Y188L	1 (0.04)	I50V	1 (0.04)
F77L	1 (0.04)	Y188C	1 (0.04)	I54L	0
Y115F	1 (0.04)	Y188H	0	I54M	0
F116Y	0	G190A	22 (0.85)	Q58E	10 (0.39)
Q151M	0	G190S	1 (0.04)	T74P	0
M184I	0	P225H	2 (0.08)	L76V	2 (0.08)
M184V	32 (1.24)			V82A	0
L210W	7 (0.27)			V82F	0
T215C	0			V82L	0
T215D	2 (0.08)			V82S	0
T215E	0			V82T	0
T215F	6 (0.23)			N83D	1 (0.04)
T215G	0			I84V	0
T215H	0			N88S	0
T215I	4 (0.16)			L90M	7 (0.27)
T215L	0				
T215N	1 (0.04)				
T215S	1 (0.04)				
T215V	0				
T215Y	13 (0.50)				
K219E	4 (0.16)				
K219Q	6 (0.23)				

Pre-treatment genotypic drug-resistance was defined as the presence of at least one amino acid substitution included in the IAS-USA mutation list of December 2010, including the revertant mutations at codon 215. Drug-classes considered were NRTIs, NNRTIs and PIs. Drug-resistance mutations: the wild-type amino acid is given, followed by the codon of the reverse-transcriptase or protease gene, followed by the amino acid substitution conferring resistance. IAS-USA, International Antiviral Society of the USA; NRTI, nucleotide reverse transcriptase inhibitors; NNRTI, nonnucleotide reverse transcriptase inhibitors; PI, protease inhibitors.

Amino acid abbreviations: A, alanine; C, cysteine; D, aspartate; E, glutamate; F, phenylalanine, G, glycine; H, histidine; I, isoleucine; K, lysine; L, leucine; M, methionine; N, asparagine; P, proline; Q, glutamine; R, arginine; S, serine; T, threonine; V, valine; W, tryptophan; Y, tyrosine.

Table 3. Overview of sensitivity analyses on virologic endpoints in the PASER-M cohort.

Summary of results

- Changing the definition of virological failure to ≥50 copies per mL showed the same results as the main analysis.
- Missing-equals-failure* analysis showed the same results as the main analysis.
- Exclusion of all participants with prior antiretroviral drug exposure from regression analysis showed the same results as the main analysis.
- Exclusion of all participants who harboured the M184V mutation (associated with recent prior use of ART) before start of treatment from regression analysis showed the same results as the main analysis.
- Participants with HIV-RNA load >1000 copies per mL but without major IAS-USA drug-resistance mutations after 12 months of ART have been classified as "No drug-resistance" in the main analysis. However, there might be a resistant virus below the sensitivity level of the population-based genotypic assay. Exclusion of this group of "possible drug-resistance" from regression analysis did not affect results.

Definition of virologic endpoint and/or patient sample	PDR groups	N	Virologic failure			
			OR (95% CI)	p	OR (95% CI)	p
Virologic failure (≥400c/mL) (main analysis)		2733				
	No PDR		1		1	
	PDR and fully-active ART		1.01 (0.55, 1.87)	0.964	0.97 (0.51, 1.58)	0.934
	PDR and partially-active ART		2.13 (1.44, 3.14)	<0.001	2.30 (1.55, 3.40)	<0.001
Virologic failure (≥50c/mL)		2096				
	No PDR		1		n/a	
	PDR and fully-active ART		1.01 (0.45, 2.25)	0.981		
	PDR and partially-active ART		2.53 (1.53, 4.18)	<0.001		
Exclusion of patients with prior antiretroviral drug exposure		2588				
	No PDR		1		1	
	PDR and fully-active ART		0.97 (0.51, 1.85)	0.919	0.91 (0.46, 1.81)	0.797
	PDR and partially-active ART		2.11 (1.36, 3.25)	0.001	2.29 (1.48, 3.55)	<0.001
Exclusion of patients harbouring a virus with the M184V mutation before ART initiation		2701				
	No PDR		1		1	
	PDR and fully-active ART		1.02 (0.55, 1.88)	0.958	0.97 (0.51, 1.84)	0.929
	PDR and partially-active ART		2.61 (1.67, 4.06)	<0.001	2.75 (1.76, 4.31)	<0.001
Exclusion of patients with "possible drug-resistance"		2690				
	No PDR		1		1	
	PDR and fully-active ART		1.01 (0.54, 1.90)	0.965	0.99 (0.52, 1.88)	0.964
	PDR and partially-active ART		2.24 (1.51, 3.29)	<0.001	2.25 (1.52, 3.33)	<0.001

Table 3 (continued)

Definition of virologic endpoint and/or patient sample	PDR groups	N	Virologic failure			
			OR (95% CI)	p	OR (95% CI)	p
Missing equals failure *		2699				
	No PDR		1		1	
	PDR and fully-active ART		0.91 (0.47, 1.74)	0.768	0.89 (0.44, 1.78)	0.742
	PDR and partially-active ART		2.31 (1.44, 9.11)	<0.001	2.36 (1.58, 3.53)	<0.001

Table shows results of multilevel logistic regression baseline models with multiple imputations for missing data. * Participants for whom no outcome HIV-RNA load result was available due to attrition (stopped ART, lost to follow-up, transferred out, died, switched to second-line ART) were classified as virologic failure (≥400 copies per mL). PDR, pre-treatment, drug-resistance; ART, antiretroviral therapy; CI, confidence interval; OR, odds ratio; n/a, not applicable

Table 4. Overview of observational cohort studies investigating the effect of pretreatment drug resistance on treatment outcome.

Panel research in context: Systematic review

Wittkop *et al.* (1)	10,056	Eurocoord and CHAIN; Genotype was not used to guide treatment selection	≥1998	Europe	Stanford algorithm: at least low-level resistance to ≥1 drug	Patients with PDR to ≥1 prescribed drug had higher risk of VL≥500 c/mL; No risk difference for patients with PDR and fully-active ART.
Bansi *et al.* (2)	935	UK-CHIC; Genotype was not used to guide treatment selection	1999-2006	UK	Stanford algorithm: GSS calculated	Patients with GSS<3 had higher risk of VL≥50 c/mL (week 52)
Chaix *et al.* (3)	350	ANRS CO 06 PRIMO; Genotype was not used to guide treatment selection	1996-2005	France	ANRS: resistant to ≥1 drug	Proportion of patients with VL<400 c/mL lower in patients with PDR (week 24)
Bannister *et al. (4).*	277	EuroSIDA; Genotype was not used to guide treatment selection	1996-2004	Europe	Stanford: at least intermediate resistance to ≥1 drug	No difference for proportion of VL <500 c/mL (week 24)
Oette *et al.* (5)	269	RESINA; Genotype was used to guide treatment selection	2001-2003	Germany	Geno2pheno	No difference for proportion of VL <50 c/mL (week 48)
Pillay *et al.* (6)	201	Seroconverters CASCADE; Genotype was not used to guide treatment selection	1996-2003	Europe	Stanford: at least intermediate resistance to ≥1 drug	No difference in time to VL suppression <500 c/mL
Peuchant *et al.* (7)	172	ANRS CO3 Aquitaine Cohort	1996-2005	France	ANRS: resistant to ≥1 drug	Slower decrease in mean viral load in patients with PDR (one month)

Studies are sorted by descending sample size. ANRS, Agence Nationale de Recherches sur le Sida et les hépatites virales; GSS, Genotypic Sensitivity Score; NRTI, nucleoside reverse transcriptase inhibitor; NNRTI, nonnucleoside reverse transcriptase inhibitor; PI, protease inhibitor; PDR, pretreatment drug-resistance; WHO, World Health Organization mutation list.

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AFRIKA

Chapter 9

Patterns of HIV-1 drug resistance after first-line antiretroviral therapy failure in six sub-Saharan African countries: implications for second-line ART strategies

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ABSTRACT

Background

HIV-1 drug resistance may limit the benefits of combination antiretroviral therapy (ART). This cohort study examined patterns of drug-resistance mutations (DRMs) in individuals with virological failure on first-line ART at 13 clinical sites in six African countries, and predicted their impact on second-line drug susceptibility.

Methods

A total of 2588 HIV-1 infected antiretroviral-naïve individuals initiated ART consisting of different nucleoside reverse transcriptase inhibitor (NRTI) backbones (zidovudine, stavudine, tenofovir or abacavir, plus lamivudine or emtricitabine) with either efavirenz or nevirapine. Population sequencing after 12 months of ART was retrospectively performed if HIV-RNA >1000 copies/mL. The 2010 IAS-USA list was used to score major DRMs. Stanford algorithm was used to predict drug susceptibility.

Results

HIV-1 sequences were generated for 142 participants who virologically failed ART, of whom 70% carried ≥1 DRM and 49% had dual-class resistance, with an average of 2.4 DRMs per sequence (range 1–8). The most common DRMs were M184V (53.5%), K103N (28.9%), G190A (14.1%), Y181C (15.5%). Thymidine analogue mutations were present in 8.5%. K65R was frequently selected by stavudine (15.0%) or tenofovir (27.7%). Among participants with ≥1 DRM, the predicted HIV-1 susceptibility was reduced in 93% for efavirenz/nevirapine, in 81% for lamivudine/emtricitabine, in 59% for etravirine/rilpivirine, in 27% for tenofovir, in 18% for stavudine, and in 10% for zidovudine.

Conclusions

Early failure detection limited the accumulation of drug-resistance. After stavudine failure in African populations, zidovudine rather than tenofovir may be preferred in second-line ART. Strategies to prevent HIV-1 resistance are a global priority.

INTRODUCTION

The rapid scale-up of access to combination antiretroviral therapy (ART) for HIV-1 infected persons in sub-Saharan Africa during the past decade, through a WHO-recommended public-health approach [1], has dramatically reduced HIV-related mortality [2]. However, the widespread use of HIV clinical staging and, if available, CD4 cell counts to diagnose ART failure in resource-limited settings, rather than routine virological monitoring, is associated with the accumulation of HIV-1 drug-resistance mutations [3-5], which may limit subsequent drug options and constitutes a source for onward transmission. Studies from the region have reported high levels of drug resistance in individuals with prolonged first-line ART failure, including complex nucleoside reverse transcriptase inhibitor (NRTI) resistance profiles, such as K65R, Q151M and thymidine analogue mutations (TAMs), in addition to highly prevalent M184V and non-nucleoside reverse transcriptase inhibitor (NNRTI) mutations [4-8].

Most reports on HIV-1 drug resistance deal with subtype B infections in developed countries. There is limited knowledge of resistance pathways in the different HIV-1 non-B subtypes and their clinical relevance [9], despite the fact that more than 90% of HIV-1 infections globally belong to non-B subtype variants [10].

The PASER-M study is a prospective cohort of HIV-1 infected individuals from 13 clinical sites in 6 sub-Saharan African countries who initiated first-line ART including different NRTI backbones (zidovudine [37%], tenofovir [34%], stavudine [27%] or abacavir [3%]) and NNRTIs (efavirenz [60%] or nevirapine [40%]), in accordance with national guidelines [11]. The present study assessed HIV-1 DRMs in participants with virological failure of different first-line regimens, and predicted viral drug susceptibility to gain insight about the optimal strategies for second-line therapy.

METHODS

Study population

The PASER-M study includes clinical sites in Kenya (2), Nigeria (1), South Africa (3), Uganda (3), Zambia (3), and Zimbabwe (1) [11]. We previously reported the participants' baseline resistance profiles [12], and the effect of pre-treatment drug resistance on the immunological and virological patient outcomes after the first year of ART [13].

For the present analysis, individuals were included if they were aged ≥18 years with HIV-1 infection and had initiated standard first-line NNRTI-based ART in accordance

with national guidelines- that is, advanced immunodeficiency (CD4 cell count < 200 cells/μL) or advanced HIV disease (WHO clinical stage 3 or 4) (13). Individuals who were previously exposed to any antiretroviral drugs for prevention and/or treatment, were excluded. Other exclusion criteria were pregnancy at study screening, or –screened in Nigeria only– HIV-2 co-infection.

Participants provided written informed consent at enrollment. The study protocol was approved by the appropriate national and local research ethics committees at all collaborating sites and the Academic Medical Center of the University of Amsterdam in The Netherlands.

Data collection

Participants were followed-up in accordance with local standard-of-care guidelines. A single-drug substitution, due to toxicity or intolerance, was not considered a regimen switch. Plasma was collected at the baseline visit and after 12 months of ART (time window, 11-15 months), and stored for retrospective assessment of HIV RNA and genotypic drug resistance. Virologic failure was defined as a plasma HIV RNA value of ≥400 copies/mL. Participants who were switched to second-line ART earlier than month 12 (owing to locally diagnosed ART failure) were not included in the month 12 summary statistics.

Virological analysis

All virological testing was conducted at 1 of 2 reference laboratories in South Africa or Uganda, as previously described [12]. Briefly, HIV RNA was determined using NucliSens EasyQ real-time assay, version 2.0 (bioMérieux, Lyon, France) or COBAS Ampliprep/COBAS Taqman assay (Roche, Branchburg, New Jersey, USA). Population-based genotyping of HIV-1 protease and reverse transcriptase was undertaken in specimens with HIV RNA >1000 copies/mL, using in-house sequencing methods. HIV-1 subtypes were inferred from the *pol* sequences using the STAR algorithm [14] and, if required, the REGA tool (version 2.0) [15]. Genotypic drug resistance was defined as the presence of ≥1 major amino acid substitution included in the International Antiviral Society-USA mutation list of December 2010 [16]. Drug-classes considered were NRTIs, NNRTIs and protease inhibitors (PIs). HIV-1 drug susceptibility for each participant was predicted using the Stanford algorithm, version 6.1.0 [17], and was categorized as susceptible, potential low-level resistance, low-level resistance, intermediate resistance, or high-level resistance. All sequences have been deposited in GenBank (accession numbers JQ480157-JQ480298).

Statistical analysis

Group comparisons for categorical data were done using chi-square or Fisher's exact test, and for continuous data using Kruskal-Wallis test, as appropriate. Mantel-Haenszel

method was used to compute weighted odds ratios (OR) that adjusted for a single confounding factor. Reported p-values are two-sided and p<0.05 was considered statistically significant. All analyses were performed using Stata version 11 (StataCorp, Texas, USA).

RESULTS

Study population and clinical outcomes

Between March 2007 and September 2009, 2588 participants were enrolled. Table 1 shows the baseline characteristics of all participants (n=2588), and of those with a resistance test result by month 12 (n=142). Participants were initiated on ART regimens containing either zidovudine (964 [37.2%]), tenofovir (867 [33.5%]), stavudine (691 [26.7%]), or abacavir (66 [2.6%]), combined with either efavirenz (1543 [59.6%]) or nevirapine (1045 [40.4%]). Baseline resistance was detected in 140 (5.4%) participants; the proportions did not differ between the initial regimens.

Table 1. Baseline characteristics of participants

Variable	Baseline	Month 12			p-value [a]
	Overall (n=2588)	Overall (n=142)	No resistance (n=42)	Resistance (n=100)	
Sex					0.13
Women	1482 (57.3)	61 (43.0)	14 (33.3)	47 (47.0)	
Men	1106 (42.7)	81 (57.0)	28 (66.7)	53 (53.0)	
Age at initiation, mean years (SD)	38.0 (9.0)	36.0 (8.5)	36.7 (7.9)	35.7 (8.8)	0.43
Country					0.79
Zambia	555 (21.5)	28 (19.7)	9 (21.4)	19 (19.0)	
South Africa	593 (22.9)	26 (18.3)	9 (21.4)	17 (17.0)	
Uganda	602 (23.3)	38 (26.8)	8 (19.1)	30 (30.0)	
Kenya	425 (16.4)	22 (15.5)	7 (16.7)	15 (15.0)	
Zimbabwe	211 (8.2)	12 (8.5)	3 (7.1)	9 (9.0)	
Nigeria	17 (25.8)	16 (11.3)	6 (14.3)	10 (10.0)	
WHO clinical stage at initiation					0.51
1 or 2	1015 (39.2)	52 (36.6)	17 (40.5)	35 (35.0)	
3 or 4	1573 (60.8)	90 (63.4)	25 (59.5)	65 (65.0)	
Baseline HIV-1 drug-resistance [b]	140 (5.4) [c]	15 (10.6)	1 (2.4)	14 (14.0)	0.10
Initial ART regimen					0.78
Efavirenz-based	1543 (59.6)	69 (48.6)	21 (50.0)	48 (48.0)	
Tenofovir-containing	720 (27.8)	33 (23.4)	10 (23.8)	23 (23.0)	

Table 1 (continued)

Variable	Baseline	Month 12			p-value [a]
	Overall (n=2588)	Overall (n=142)	No resistance (n=42)	Resistance (n=100)	
Stavudine-containing	430 (16.6)	11 (7.8)	5 (11.9)	6 (6.0)	
Zidovudine-containing	342 (13.2)	23 (16.2)	6 (14.3)	17 (17.0)	
Abacavir-containing	51 (2.0)	2 (1.4)	0 (0.0)	2 (2.0)	
Nevirapine-based	1045 (40.4)	73 (51.4)	21 (50.0)	52 (52.0)	
Zidovudine-containing	622 (24.0)	48 (33.8)	13 (31.0)	35 (35.0)	
Stavudine-containing	261 (10.1)	9 (6.3)	4 (9.5)	5 (5.0)	
Tenofovir-containing	147 (5.7)	14 (9.9)	3 (7.1)	11 (11.0)	
Abacavir-containing	15 (0.6)	2 (1.4)	1 (2.4)	1 (1.0)	
CD4 cell count, median cells/μL (IQR)	133 (62-204) [d]	120 (52-198	157 (136)	108 (127.5)	0.002
Plasma HIV RNA, median $\log_{10}$ c/mL (IQR)	5.00 (4.38-5.59) [e]	5.23 (4.53-5.67)	5.18 (1.31)	5.24 (.99)	0.19
HIV-1 subtypes [f]					0.14
A	611 (24.9)	37 (26.1)	14 (33.3)	23 (23.0)	
C	1329 (54.2)	69 (48.6)	22 (52.4)	47 (47.0)	
D	276 (11.3)	19 (13.4)	1 (2.4)	18 (18.0)	
CRF02_AG	116 (4.7)	6 (4.2)	2 (4.8)	4 (4.0)	
Other	118 (4.8)	11 (7.8)	3 (7.1)	8 (8.0)	

Baseline characteristics of all participants at baseline (n=2588) and of those who had a genotypic resistance test results by month 12 (n=142). Data are no. (%) unless stated otherwise. [a] P values for comparison between no resistance vs resistance outcome groups. [b] Defined as at least 1 drug-resistance mutation of the 2010 International Antiviral Society–USA list. [c] Data available for n=2442. [d] Data available for n=2578. [e] Data available for n=2564. [f] Data available for n=2450. ART, antiretroviral therapy; HIV-1, human immunodeficiency virus type 1; IQR, interquartile range; SD, standard deviation; WHO, World Health Organization.

A total of 2132 (82.4%) participants were retained in care up to 12 months of follow-up, of whom 2128 (82.2%) were still on first-line ART and 4 (0.2%) were switched to second-line therapy earlier than month 12 due to locally diagnosed ART failure. The remaining 456 (17.6%) participants were not retained, because they died (190 [7.3%]), were lost to follow-up (200 [7.7%]), transferred out (61 [2.4%]), or discontinued ART (5 [0.2%]) (figure 1).

HIV RNA by month 12 was assessed for 94.6% (2014 of 2128) of participants who were still on first-line ART. For all who initiated first-line ART, 70.3% (1820 of 2588; 95%CI 68.5-72.1) achieved viral suppression, and for those who had a 12-month HIV RNA result 90.4% (1820 of 2014; 95%CI 89.0-91.6) achieved viral suppression (figure 1).

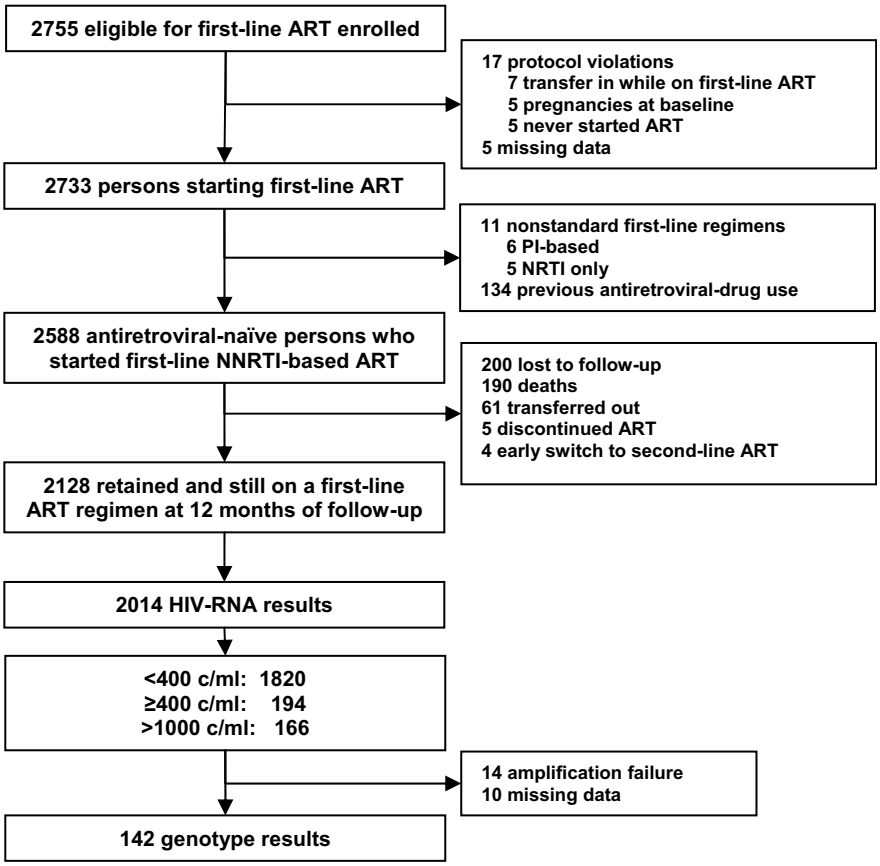

Figure 1. Study profile

ART, antiretroviral therapy; PI, protease inhibitor; NRTI, nucleoside reverse transcriptase inhibitor; NNRTI, non-NRTI.

Drug-resistance mutations by drug regimen

Of the 166 participants with HIV RNA >1000 copies/mL by month 12, sequence results were available for 142 (85.5%); 14 specimens failed to amplify, and results were missing for 10 specimens. One hundred (70.4%, 95%CI 62.2-77.8) sequences harboured ≥1 DRM and 42 (29.6%) did not harbour any DRMs. The average number of DRMs (any class) per sequence was 2.4, with a range of 1–8. Detected DRMs were associated with NRTIs (82 [57.8%]), NNRTIs (86 [60.6%]) (figure 2). Dual-class resistance to NRTIs and NNRTIs was detected in 69 (48.6%) participants. Combinations of DRMs included M184V and NNRTI (64 [45.1%]), M184V and TAMs (10 [7.6%]), TAMs and NNRTI (8 [5.6%]), and M184V, TAMs, and NNRTI (7 [4.9%]) (figure 2). In 2 (1.4%) participants, DRMs known to be associated with PIs were observed (both tipranavir-associated Q58E); no triple-class resistance was detected. Exclusion of the 16 (9.6%) participants who had ≥1 single-drug substitutions

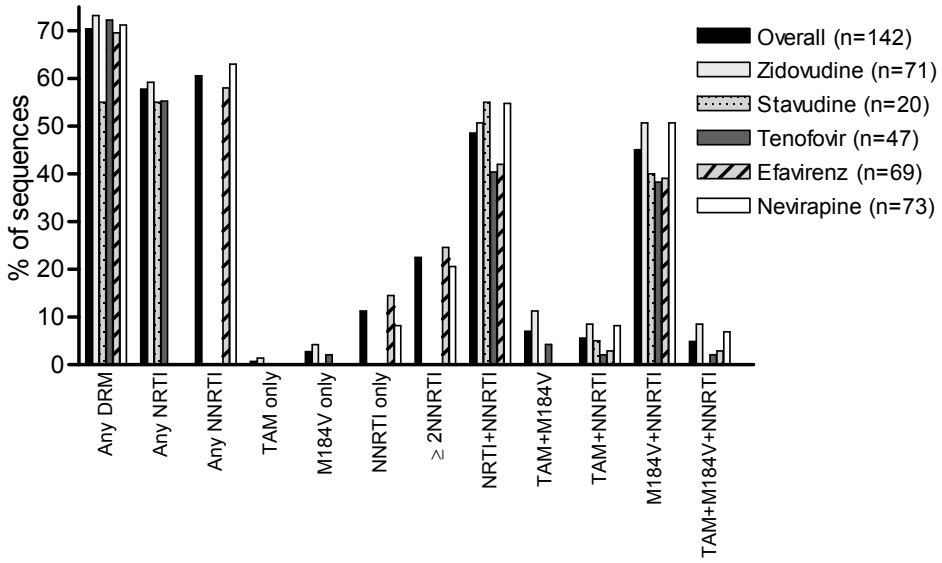

Figure 2. Frequencies of any drug resistance according to drug class and permutations by nucleoside reverse transcriptase inhibitor (NRTI) and by non-nucleoside reverse transcriptase inhibitor (NNRTI).
Figure shows major International Antiviral Society–USA drug-resistance mutations associated with NRTIs and NNRTIs in participants experiencing virological failure. The legend includes the number of patients exposed to each antiretroviral drug. DRM, drug-resistance mutation; TAM, thymidine analogue mutation.

during the first-year of ART did not significantly change the frequencies of the DRMs (not shown).

Of the NRTI-associated DRMs (figure 3), M184V was the most frequent (76 [53.5%]), followed by K65R (17 [12.0%]) and any TAM (12 [8.5%]). In 9 (6.3%) participants, ≥2 TAMs were detected and in 3 (2.1%) participants, ≥3 TAMs were detected. TAM-1 mutations included M41L and T215 F/Y (L210W not observed); TAM-2 mutations included D67N, K219E and K70R (K219Q not observed). In participants who failed regimens containing zidovudine, stavudine or tenofovir, ≥1 TAM was detected in 12.7% (9 of 71), 5.0% (1 of 20), and 4.3% (4 of 47), respectively; K65R in 0% (0 of 71), 15.0% (3 of 20) and 27.7% (13 of 47), respectively. Q151M was not detected.

Of the NNRTI-associated DRMs (figure 3), K103N (41 [28.9%]) was the most frequent, followed by Y181C (22 [15.5%]) and G190A (20 [14.1%]). In participants failing an efavirenz-based or nevirapine-based regimen, NNRTI-associated DRMs were detected in 58.0% (40 of 69) and 63.0% (46 of 73), respectively (p=0.5); K103N in 33.3% (23 of 69) and 24.7% (18 of 73), respectively (p=0.3); Y181C in 7.3% (5 of 69) and 23.3% (17 of 73) participants, respectively (p=0.008); and V106M in 20.3% (14 of 69) and 1.4% (1 of 73), respectively (p<0.001). V106A only occurred after nevirapine exposure. Sixteen (11.3%) participants

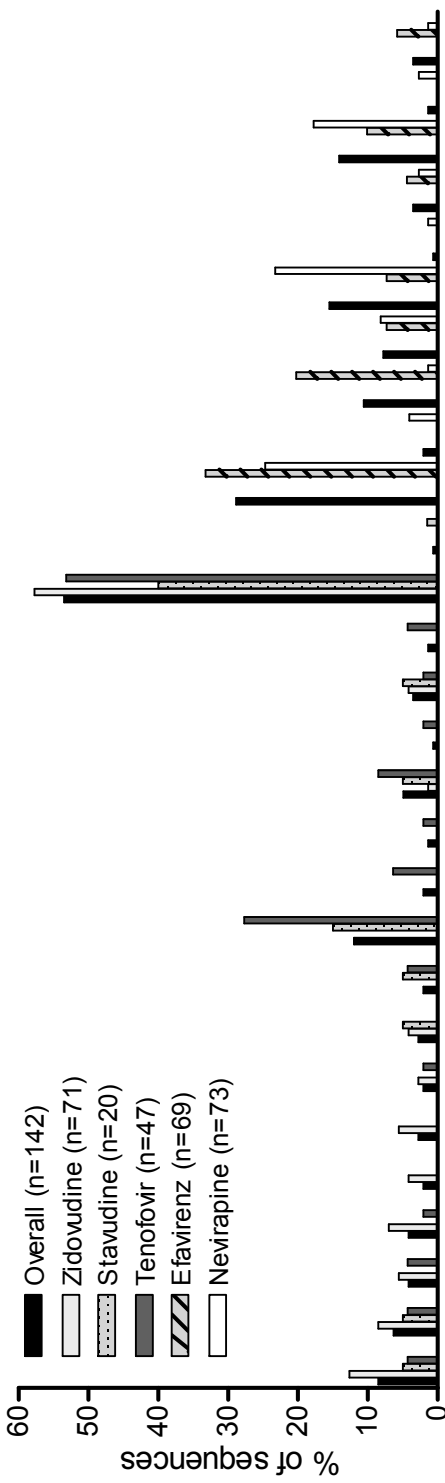

Figure 3. Frequencies of individual drug-resistance mutations by nucleoside reverse transcriptase inhibitor (NRTI) and by non-nucleoside reverse transcriptase inhibitor (NNRTI).

Figure shows major International Antiviral Society–USA drug-resistance mutations associated with NRTIs and NNRTIs in participants experiencing virological failure. The legend includes the number of patients exposed to each antiretroviral drug. TAM, thymidine analogue mutation.

harbored only DRMs associated with NNRTIs, and 33 (22.5%) participants harbored ≥2 NNRTI-associated DRMs. K103N occurred as the only NNRTI mutation in 23 of 41 participants (56.1%), and occurred in combination with other NNRTI mutations in 18 participants (with Y181C [7], P225H [3], G190A [2], V106M [1], M230L [1], V106M+Y181C [1], Y181C+G190A [1], P225H+M230L [1], L100I+P225H [1]).

Compared with participants with virological failure by month 12 who did not harbour any DRM, those with ≥1 DRM had a marginally lower median HIV RNA (4.33 vs 4.71 $\log_{10}$ copies/mL; p=0.04); this difference was least pronounced for NNRTI resistance (4.30 vs. 4.55 $\log_{10}$ copies/mL, p=0.05) and most pronounced for M184V (4.21 vs. 4.59 $\log_{10}$ copies/mL, p=0.02).

Of the 142 participants with a genotype result by month 12, 135 had a baseline genotype result (7 baseline specimens not collected); of these, 14 (10.4%) participants harboured ≥1 DRM before start of ART. Of the DRMs detected by month 12, 96% were newly acquired during the first year of ART.

HIV-1 subtype diversity

Among the 142 participants who had a resistance test result by month 12, HIV-1 subtype C was most commonly identified, followed by A, D, A/G recombinant, and other subtypes/recombinants (table 1). The subtype distribution did not differ significantly between those with or without DRMs (p=0.14). The DRMs according to subtype are shown in figure 4. K65R was more frequent in subtype C (12 [17.4%]) than non-C (5 [6.9%]) (p=0.05). After adjusting for differential tenofovir and stavudine exposure, the association with subtype was not significant: OR for C vs non-C (by Mantel-Haenszel method) 1.27; 95%CI 0.41-3.93; p=0.68).

Compared with subtype C (13 [18.8%]), K103N occurred more frequently in D (10 [52.6%]; p=0.003) and A (13 [35.1%]; p=0.06), but did not differ between A and D (p=0.21). After adjusting for differential efavirenz and nevirapine exposure, K103N remained significantly associated with subtype: OR for C vs non-C (by Mantel Haenszel method) 0.33; 95%CI 0.15-0.75; p=0.005, and OR for D vs non-D 3.40; 95%CI 1.21-9.58; p=0.014. Frequencies of other DRMs, except for V106M, which was exclusively observed in subtype C, did not differ between HIV-1 subtypes (figure 4).

Predicted genotypic drug susceptibility

Of the viruses harboring ≥1 DRM (n=100), the predicted HIV-1 susceptibility to lamivudine and emtricitabine was reduced in the majority (81%), due to the high frequency of M184V (figure 5). For the other NRTIs, reduced HIV-1 susceptibility was predicted for

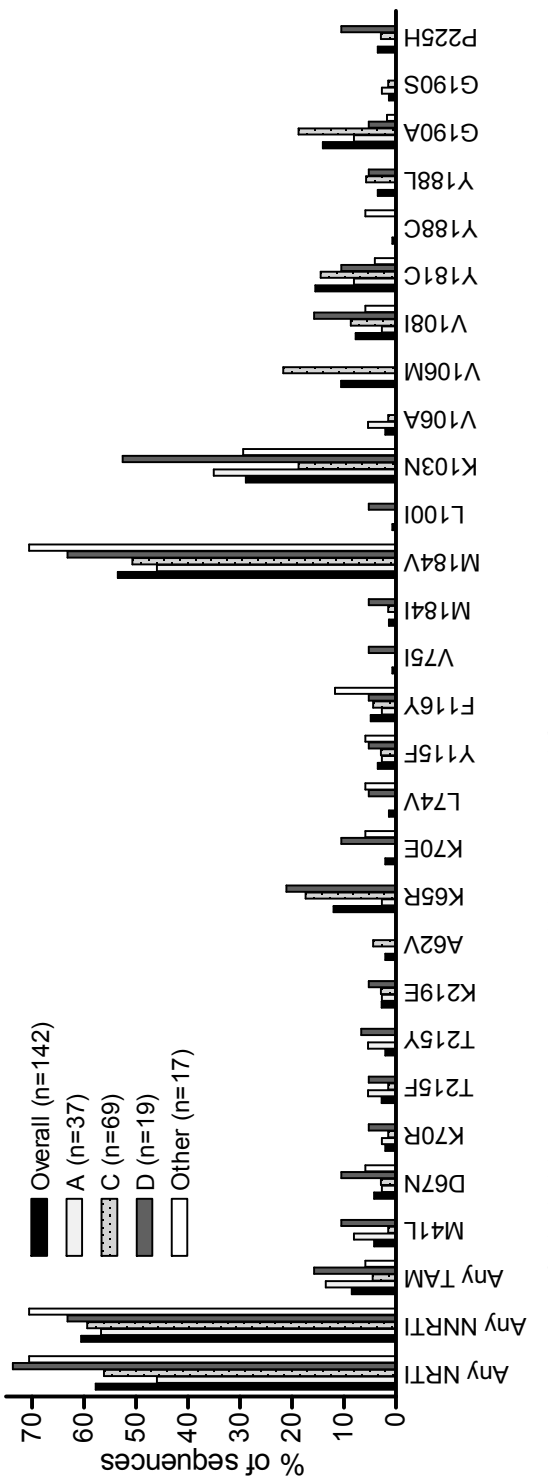

Figure 4. Frequencies of individual drug-resistance mutations by human immunodeficiency virus type 1 (HIV-1) subtype.
Figure shows major International Antiviral Society–USA drug-resistance mutations associated with nucleoside reverse transcriptase inhibitors (NRTIs) and non-nucleoside reverse transcriptase inhibitors (NNRTIs) in the participants experiencing virological failure. The legend includes the number of patients infected with each HIV-1 subtype. TAM, thymidine analogue mutation.

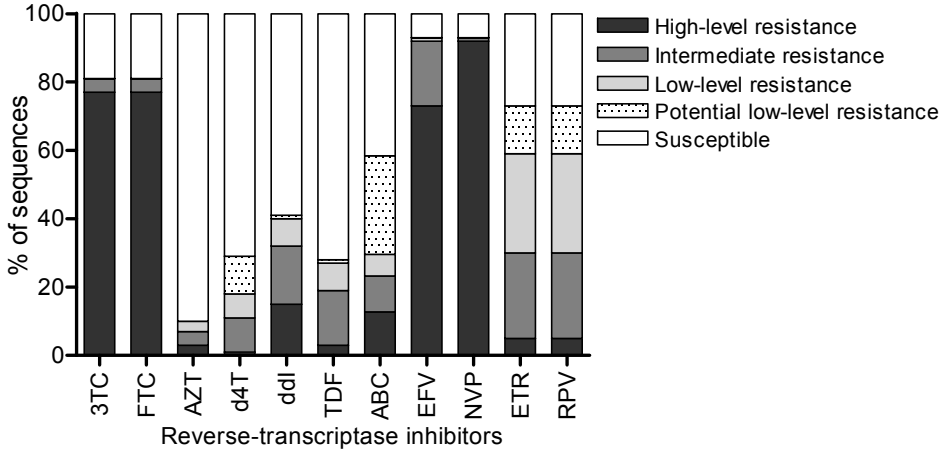

Figure 5. Predicted viral susceptibility to reverse transcriptase inhibitors.
Figure includes the viruses that harbored ≥1 drug-resistance mutation (n= 100). Genotypic drug susceptibility was predicted using the Stanford HIVdb algorithm (version 6.1.0). 3TC, lamivudine; ABC, abacavir; AZT, zidovudine; d4T, stavudine; ddI, didanosine; EFV, efavirenz; ETR, etravirine; FTC, emtricitabine; NVP, nevirapine; RPV, rilpivirine; TDF, tenofovir.

abacavir in 42% of sequences, didanosine in 40%, tenofovir in 27%, stavudine in 18%, and zidovudine in 10%. For the NNRTIs, reduced HIV-1 susceptibility was frequently predicted for the first-generation drugs efavirenz and nevirapine (93%) and less frequently for the second-generation drugs etravirine and rilpivirine (59%). High-level resistance was most frequently predicted for nevirapine (92%), followed by lamivudine/ emtricitabine (77%) and efavirenz (73%), but was uncommon for etravirine and rilpivirine (5%). Reduced HIV-1 susceptibility was not predicted for any of the ritonavir-boosted PIs.

DISCUSSION

This multi-country cohort study examined HIV-1 resistance mutation patterns in African patients with virological failure on different first-line ART regimens, and predicted their impact on second-line drug susceptibility. After the first year of ART, 70% of those with virological failure had a virus with ≥1 DRM, and dual-class resistance was observed in 49%. Nearly all (96%) DRMs detected by month 12 were newly acquired in the first year of ART. Previous studies from the region have reported higher frequencies (83-93% of those with virological failure) and complexity of DRM patterns in persons with prolonged ART failure, in the absence of viral load monitoring [4-8]. By contrast, in the present study, routine viral load testing after 12 months of ART enabled the relatively early detection of virological ART failure, which, to some extent, may have prevented the accumulation of resistance. Nonetheless, observed resistance patterns for this cohort were more

extensive than for cohorts that received intensive virological monitoring in South Africa [18] and resource-rich countries [19, 20]. Therefore, our data underscore the importance of implementing routine viral load monitoring in ART programs in sub-Saharan Africa to prevent drug-resistance accumulation [3-5]. The presence of DRMs was associated with lower HIV RNA values, which results from the reduced fitness of resistant variants.

In patients who failed a tenofovir-containing regimen, the K65R mutation, which is tenofovir's signature mutation, was commonly observed. Notably, K65R, rather than TAMs, was also frequently selected after stavudine failure. This finding concurs with previous clinical studies that reported high rates of K65R in subtype C–infected Africans who failed stavudine-containing regimens [4, 5, 7], and contrasts with the low frequencies of K65R observed in subtype-B-infected individuals from developed countries [21, 22]. K65R and TAMs represent antagonistic pathways of NRTI resistance [23], and K65R confers cross-resistance to all NRTIs except zidovudine.

Several mechanistic studies have demonstrated *in vitro* that the K65R mutation, which confers broad cross-resistance to the NRTI class, develops more readily in HIV-1 subtype C, the predominant subtype in sub-Saharan Africa, than in subtype B [24-29]. In our cohort, which included only non-B subtypes, K65R was detected more frequently in subtype C than in subtypes A or D. However, after adjusting for differential stavudine and tenofovir use across the subtypes, the association was not statistically significant. This discrepancy with the published literature is most likely explained by the limited statistical power of the present study to assess the effects of subtype on the rates of individual DRMs. Nonetheless, overall, our data support the conclusion that K65R is frequently selected after stavudine or tenofovir use in African populations receiving first-line ART. Therefore, from a virological perspective, zidovudine rather than tenofovir (as currently recommended by the WHO guidelines [30]), may be preferred in second-line therapy for non-B subtype infected patients who fail a stavudine-containing first-line regimen in the absence of stringent viral load and resistance monitoring. This scenario would warrant enhanced toxicity monitoring, particularly for zidovudine-associated anemia [31].

The WHO advises that stavudine be phased out of first-line regimens due to its serious adverse effects, to be replaced with either tenofovir or zidovudine [30]. Dose reduction (from 40 mg to 30 mg, and even 15-20 mg per day) has been suggested to limit the mitochondrial toxicities from stavudine [32]. However, suboptimal dosing of stavudine might in theory result in even more frequent emergence of K65R, particularly when used as part of NNRTI-based regimens. Emerging K65R will compromise future NRTI backbones, and clinical trials are ongoing to establish the effectiveness of ("simplified") PI-based second-line therapy without an effective NRTI-backbone [33].

For second-line ART, WHO recommends a ritonavir-boosted PI (preferably lopinavir or atazanavir), with a dual backbone of 2 new or recycled NRTIs, that is, tenofovir plus lamivudine/emtricitabine (after use of stavudine or zidovudine in first-line), or zidovudine plus lamivudine (after use of tenofovir in first-line) [30]. In our cohort, HIV-1 susceptibility to all PIs was preserved for all participants; zidovudine and tenofovir susceptibility was preserved in the majority of patients who failed first-line ART (90% and 73%, respectively). This means that the majority of patients who are switched empirically to second-line PI-based therapy will receive at least two active drugs. Preliminary studies in Africa have indeed suggested high chance of viral re-suppression with empirically prescribed second-line regimens [34-38].

The second-generation NNRTIs rilpivirine or etravirine are being considered as second-line drugs in resource-limited countries in patients previously exposed to efavirenz or nevirapine, because of their different resistance profiles and suggested high genetic barrier. Susceptibility to the second-generation NNRTIs is preserved in viruses containing the K103N mutation, preferentially selected by efavirenz, but susceptibility is reduced in the presence of Y181C, preferentially selected by nevirapine [39]. In our cohort, viral susceptibility to the second-generation NNRTIs was already reduced in 59% of participants who had ≥1 DRM. Given that NRTI and NNRTI resistance mutations rapidly accumulate in the absence of viral load monitoring [4, 5], second-line regimens based on 2 NRTIs and a second-generation NNRTI are therefore unlikely to be effective. Instead, use of second-generation NNRTIs as part of first-line ART in resource-limited countries merits further evaluation.

In our cohort, K103N occurred more frequently in subtype D infections than in subtype C infections. By contrast, previous studies in women and infants after the use of single-dose nevirapine to prevent vertical HIV-1 transmission have suggested that K103N accumulates faster in subtype C than D, and faster in D than A [40-42]. Additional studies are needed to elucidate the virological mechanisms for different subtypes.

This study has some limitations. Population-based sequencing is not able to detect minority resistant viral strains, thus potentially underestimating resistance [43]. The lack of frequent serial blood sampling after ART initiation precluded a more detailed analysis of the evolution of DRMs after ART initiation.

The number of HIV-1 infected people accessing ART in resource-limited countries is projected to increase further in view of the goal of universal access [2] and, particularly, the earlier start of ART [44] and emerging treatment-for-prevention [45]. Before widely implementing such strategies, mathematical modelling as well as empirical data are

urgently needed to investigate their acceptability, adherence as well as the possible consequences for the emergence and spread of drug resistance.

In conclusion, regular viral load monitoring aimed at early failure detection may limit the accumulation of HIV-1 drug-resistance. The strengthening of national HIV treatment programs through robust supply chains, routine viral load monitoring and improved access to alternative drug regimens in sub-Saharan Africa to prevent drug resistance, is an urgent priority. Zidovudine rather than tenofovir may be the preferred second-line NRTI after first-line stavudine failure. The high frequencies of Y181C and accumulated NNRTI mutations limit the effectiveness of second-generation NNRTIs for use in second-line ART.

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Contributors

RLH, CLW, WS, TFRW and RS designed the study and developed the protocol. RLH and KCES set up the study and trained and supervised study workers. CK, MS, FC, MB, KM, MW, and AO established the cohort and supervised data collection. CLW, CK, and WS supervised the laboratory testing. RLH conceived and undertook the data analyses, and drafted the manuscript. KCES, AMW, CLW and RS helped interpret the data and reviewed the manuscript. All authors reviewed and approved the final version of the manuscript.

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AFRIKA

Chapter 10

Unnecessary antiretroviral treatment switches and accumulation of HIV resistance mutations; two arguments for viral load monitoring in Africa

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ABSTRACT

Objectives

This study aimed to investigate the consequences of using clinico-immunological criteria to detect antiretroviral treatment (ART) failure and guide regimen switches in HIV-infected adults in sub-Saharan Africa. Frequencies of unnecessary switches, patterns of HIV drug resistance, and risk factors for the accumulation of nucleoside reverse transcriptase inhibitor (NRTI)–associated mutations were evaluated.

Methods

Cross-sectional analysis of adults switching ART regimens at 13 clinical sites in 6 African countries was performed. Two types of failure identification were compared: diagnosis of clinico-immunological failure without viral load testing (CIF only) or CIF with local targeted viral load testing (targeted VL). After study enrollment, reference HIV RNA and genotype were determined retrospectively. Logistic regression assessed factors associated with multiple thymidine analogue mutations (TAMs) and NRTI cross-resistance (≥2 TAMs or Q151M or K65R/K70E).

Results

Of 250 patients with CIF switching to second-line ART, targeted VL was performed in 186. Unnecessary switch at reference HIV RNA <1000 copies per milliliter occurred in 46.9% of CIF only patients versus 12.4% of patients with targeted VL ($P < 0.001$). NRTI cross-resistance was observed in 48.0% of 183 specimens available for genotypic analysis, comprising ≥2 TAMs (37.7%), K65R (7.1%), K70E (3.3%), or Q151M (3.3%). The presence of NRTI cross-resistance was associated with the duration of ART exposure and zidovudine use.

Conclusions

Clinico-immunological monitoring without viral load testing resulted in frequent unnecessary regimen switches. Prolonged treatment failure was indicated by extensive NRTI cross-resistance. Access to virological monitoring should be expanded to prevent inappropriate switches, enable early failure detection and preserve second-line treatment options in Africa.

INTRODUCTION

Over the past decade, there has been an unparalleled effort to provide access to antiretroviral treatment (ART) for HIV-infected individuals in sub-Saharan Africa, the region with the highest HIV burden [1]. Although short-term outcomes of first-line ART have been favorable [2], extended follow-up data are still limited. A proportion of patients receiving ART will inevitably experience treatment failure, putting them at increased risk of HIV-related morbidity and mortality. Recent guidance from the World Health Organization (WHO) recommends viral load determination, if feasible, to improve the identification of treatment failure [3]. Due to financial and logistical constraints in resource-limited settings, however, access to this expensive and technically demanding test is limited. Therefore, as a substitute, WHO-recommended clinical criteria and CD4 cell counts are commonly used by clinicians to diagnose ART failure and guide switches to second-line regimens.

Several studies in African countries, however, have shown poor association of clinico-immunological criteria with virological failure in patients on first-line ART [4-7]. In absence of viral load testing, incorrectly diagnosed or presumed virological failure may result in inappropriate switches to more expensive and toxic second-line regimens. Additionally, delayed failure detection and continuation of a failing regimen can result in the selection of viruses with extensive resistance to antiretroviral (ARV) drugs [8]. In particular, the accumulation of mutations associated with cross-resistance within the nucleoside reverse transcriptase inhibitor (NRTI) drug class may compromise the effectiveness of standard second-line regimens, which are based on a dual backbone of new or recycled NRTIs and a ritonavir-boosted protease inhibitor (PI).

This multicenter study, conducted in a collaborative network of 13 ART sites in 6 African countries [9], aimed to investigate the consequences of using clinicoimmunological criteria to detect treatment failure and guide regimen switching. To this end, we sought to evaluate frequencies of unnecessary switches to second-line regimens, patterns of HIV drug resistance in patients failing first-line ART, and risk factors for the accumulation of NRTI-associated mutations.

METHODS

Study design and population

The PharmAccess African Studies to Evaluate Resistance Monitoring (PASER-M) study is a multicenter prospective observational cohort of HIV-1–infected adults who receive

ART in routine circumstances at 13 clinical sites in Kenya, Nigeria, South Africa, Uganda, Zambia, and Zimbabwe. Collaborating sites were selected to represent a variety of ART programs in terms of site administration (government, nongovernment, and private), ART experience, and geography. Sites were considered eligible to collaborate in PASER-M if they had accruing ART programs and minimum standards of administration and laboratory capacity. Eight sites had access to viral load testing, whereas 5 sites used clinico-immunological monitoring only. Cohort and site characteristics have been profiled previously [9]. The study was approved by local Ethics Committees and the Academic Medical Center Institutional Review Board. Written informed consent was obtained from all participants before the start of study procedures.

For the current cross-sectional analysis, PASER-M study participants were included if they were switched to second-line ART by their treating clinician, regardless of whether clinical, immunological, and/or virological criteria had been used to diagnose treatment failure. Exclusion criteria were a positive pregnancy test at study screening, and, in Nigeria, HIV-2 co-infection. We compared patients based on the type of failure identification used by the treating clinician: diagnosis of CIF in absence of viral load testing ("CIF only" group) or CIF with local targeted viral load (VL) testing ("targeted VL" group). In the CIF only group, the clinician's decision to switch was based on a new WHO clinical stage 3 or 4 condition or immunological deterioration, as defined by a CD4 cell count fall to pretreatment value, CD4 cell count decrease of 50% or persistent CD4 levels <100 cells per cubic millimeter. In the targeted VL group, treating clinicians had access to a local real-time HIV RNA test result to confirm suspected treatment failure based on clinical and immunological information. Drug changes because of side effects or toxicity were not considered regimen switches and were excluded from analysis. Demographic and clinical information were collected using standard case report forms.

Laboratory procedures

Routine laboratory results including CD4 cell count and HIV RNA were obtained from local laboratory records. Before switch to second-line ART, an additional phlebotomy was performed and EDTA anti-coagulated plasma specimens were stored at −80°C and batch-shipped to 2 reference laboratories in South Africa (Genotyping Unit, Department of Molecular Medicine and Hematology, University of the Witwatersrand) and Uganda (Genotyping Laboratory, Joint Clinical Research Centre) for retrospective determination of reference HIV RNA and genotypic resistance testing on all specimens with HIV RNA >1000 copies per milliliter. The Ugandan laboratory performed testing for the Ugandan sites and the South African laboratory performed testing for all other sites. Both laboratories participated in external quality assessment schemes for HIV RNA and genotypic testing. Tests performed at the reference laboratory are denoted "reference HIV-RNA"

throughout this report, and results obtained from the clinic are referred to as "local HIV RNA". For patients in the targeted VL group, both local and reference HIV RNA results were available. The reference HIV RNA was considered the gold standard.

The South African laboratory used the NucliSens EasyQ real-time assay version 2.0 (bioMérieux, Lyon, France) for reference HIV RNA determination and an in-house sequencing method encompassing the whole of protease and codons 1–300 of reverse transcriptase with an ABI Prism 3730 Genetic Analyzer Genetic Analyzer (Applied Biosystems, Foster City, CA) [10]. Sequences were assembled and manually edited using Sequencher v4.8 (Genecodes, Ann Arbor, MI). The Ugandan laboratory used the COBAS Ampliprep/ COBAS Taqman HIV-1 test (Roche, Branchburg, NJ) for HIV RNA determination and an in-house sequencing method encompassing the whole of protease and codons 1–300 of reverse transcriptase with a Beckman Coulter CEQ 8000 analyzer (Beckman Coulter Inc, Fullerton, CA) [11]. Sequences were assembled and manually edited using BioEdit version 7.0.9.0. All final sequences were submitted to the ViroScore database (Advanced Biological Laboratories SA, France) for quality control and data storage. Drug resistance mutations were scored according to the 2009 International AIDS Society–USA list of December 2009 [12]. HIV with genetic mixtures of wild-type and mutant sequences at amino acid sites that code for drug resistance mutations were considered resistant. Subtypes were determined using the REGA HIV-1 subtyping algorithm version 2.0 [13] and additional STAR genotype analysis if required [14].

Statistical analysis

An unnecessary switch to second-line ART was defined using 3 reference HIV RNA cut-offs: <400 copies per milliliter, <1000 copies per milliliter, and the WHO-recommended threshold of <5000 copies per milliliter [3]. The sensitivity, specificity, positive predictive ratio (PPV) and negative predictive values of the type of failure identification used (CIF only vs. targeted VL) were calculated with 95% confidence intervals using 2×2 contingency tables, compared with the reference HIV RNA load. The positive and negative likelihood ratios were calculated from the sensitivity and specificity. Patient characteristics were compared using the χ^2 test for categorical data and the Wilcoxon rank-sum test for continuous data. NRTI cross-resistance was defined as the presence of ≥2 thymidine analogue mutations (TAMs), the tenofovir (TDF)-associated mutations K65R or K70E, or the Q151M complex. Univariate and multivariate logistic regression was performed to identify factors associated with the following outcomes: ≥2 TAMs, NRTI cross-resistance, or selected single mutations. Explanatory variables included in the analysis were sex, age, type of failure identification (CIF only vs. targeted VL), WHO clinical stage, CD4 cell count, and HIV-RNA load at time of treatment failure, HIV-1 subtype, total duration of previous ARV exposure and type of prior non-nucleoside reverse transcriptase inhibitors

(NNRTIs) or NRTIs. Explanatory variables that were associated with the outcome variables ($P < 0.10$) in univariate analysis were forwarded to a multivariate prediction model, using a step forward procedure. Results were expressed as odds ratios (ORs) with 95% confidence intervals and P values with $P < 0.05$ regarded statistically significant. Analyses were performed using the statistical software package Stata version 10 (StataCorp LP, TX).

RESULTS

Patient characteristics

Between March 2007 and September 2009, 250 patients with CIF were switched to a second-line regimen (figure 1). The treating clinician had diagnosed CIF only in 64 (25.6%) patients and used targeted VL testing in 186 (74.4%) patients. Patients originated from

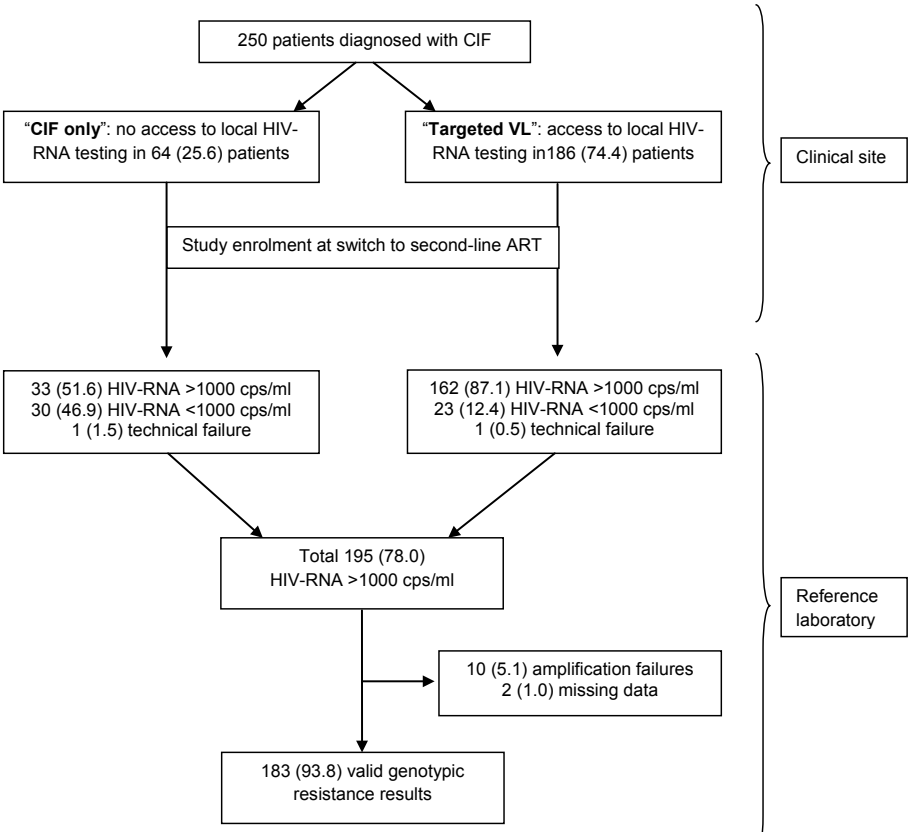

Figure 1. Flow chart of type of failure identification, study enrollment, and laboratory testing.
Data are n (%) of patients. CIF, clinico-immunological failure; VL, viral load; ART, antiretroviral treatment.

Uganda (n = 78, 31.2%), South Africa (n = 66, 26.4%), Kenya (n = 37, 14.8%), Nigeria (n = 32, 12.8%), Zambia (n = 27, 10.8%), and Zimbabwe (n = 10, 4%). Patient characteristics at switch to second-line ART are summarized in table 1. Advanced HIV disease (WHO clinical stage 3 or 4) and severe immunodeficiency (CD4 count <100 cells/mm^3) was more frequently observed in patients with CIF only (P = 0.007 and P = 0.021, respectively). The median reference HIV RNA $\log_{10}$ level was 3.3 [interquartile range (IQR): 1.4–4.4] in patients with CIF only and 4.4 (IQR: 3.7–5.0) in patients with targeted VL (P < 0.001).

The median duration of the first-line ART was 28.3 months in patients with CIF only and 25.3 months in patients with targeted VL (P = 0.310). Most patients (n = 236, 95.5%)

Table 1. Patient Characteristics at Switch to Second-Line Treatment, by Type of Identification of Failure

	Overall (n = 250)	**CIF Only (n = 64)**	**Targeted VL (n = 186)**	***P****
Women (%)	125 (50)	36 (56.3)	89 (47.8)	0.246
Median age (IQR) (yrs)	38.4 (34.2–45.0)	39.5 (34.9–45.9)	38.0 (33.8–44.9)	0.305
Median duration of prior ART regimen (IQR) (months)	26.1 (14.3–43.7)	28.3 (17.0–47.4)	25.25 (13.9–43.2)	0.310
ART regimen at failure (%)				0.016
ZDV + 3TC + NVP	67 (26.8)	15 (23.4)	52 (28.0)	
ZDV + 3TC + EFV	40 (16.0)	9 (14.1)	31 (16.7)	
D4T + 3TC + NVP	52 (20.8)	22 (34.4)	30 (16.1)	
D4T + 3TC + EFV	47 (18.8)	10 (15.6)	37 (19.9)	
TDF + FTC + NVP	12 (4.8)	1 (1.6)	11 (5.9)	
TDF + FTC + EFV	12 (4.8)	0	12 (6.5)	
NNRTI-based, other	6 (2.4)	1 (1.6)	5 (2.7)	
Triple NRTI	4 (1.6)	3 (4.8)	1 (0.5)	
PI-based	7 (2.8)	1 (1.6)	6 (3.2)	
WHO clinical stage 3 (%)	78 (31.2)	27 (42.9)	51 (27.4)	0.031
WHO clinical stage 4 (%)	40 (16.0)	12 (19.1)	28 (15.1)	
Median CD4 cell count (IQR) (cells/mm^3)	124 (59.5–200)	119 (58–177)	125.5 (61.5–215)	0.195
<100 (n, %)	103 (41.5)	30 (46.8)	73 (39.7)	0.021
100–200 (n, %)	82 (33.1)	26 (40.6)	56 (30.4)	
>200 (n, %)	63 (25.4)	8 (12.5)	55 (29.9)	
Median HIV RNA (IQR) ($\log_{10}$ c/mL)	4.2 (3.3–5.0)	3.3 (1.4–4.4)	4.4 (3.7–5.0)	<0.001
<1000 (n, %)	53 (21.2)	30 (46.9)	23 (12.4)	<0.001
1000–5000 (n, %)	25 (10.0)	2 (3.1)	23 (12.4)	
5000–10000 (n, %)	19 (7.6)	3 (4.7)	16 (8.6)	
>10000 copies/mL (%)	151 (60.4)	28 (43.8)	123 (66.1)	

Percentages are shown in parentheses and IQRs in square brackets. *Categorical data were compared using the χ^2 test; continuous were compared using the Wilcoxon rank-sum test. 3TC, lamivudine; FTC, emtricitabine.

used a NNRTI triple regimen at time of treatment failure. Others had received PI-based regimens (n = 7, 2.8%) or triple nucleoside regimens (n = 4, 1.6%). The most frequently used NRTI was zidovudine (ZDV, n = 107, 43.3%), followed by stavudine (D4T, n = 99, 40.1% and TDF (n = 24, 9.7%). The prior use of D4T was more common in patients with CIF only ($P = 0.049$), and TDF was used more frequently in patients with targeted VL ($P = 0.011$). In addition to first-line ART, 9 (4.8%) patients with targeted VL had a history of non-suppressive ARV use, either as mono-therapy (n = 2) or dual therapy (n = 3) or for the prevention of mother to child transmission (n = 4).

Diagnostic performance of treatment failure criteria

The median difference between the local and reference HIV RNA was 0.032 $\log_{10}$ copies (IQR: −0.23 to 0.52, $P = 0.0101$). Table 2 summarizes the performance of CIF only and targeted VL criteria to identify virological failure. Using the HIV RNA cut-off of <5000 copies per milliliter (table 2), unnecessary switches occurred in 78 (31.2%) patients: 32 (50.0%) in the CIF only group versus 46 (24.7%) in the targeted VL group ($P < 0.001$). CIF only criteria had a sensitivity of 18.2%, a specificity of 59%, and a PPV of 49.2%. Using the HIV RNA cut-off of <1000 copies per milliliter (table 2), unnecessary switches occurred

Table 2. Performance of CIF only* and CIF with targeted VL† criteria to identify virological failure at switch to second-line treatment

	Sensitivity (%)‡§	Specificity (%)‡§	PPV (%)‡§	NPV (%)‡§	PLR	NLR
Compared with who definition for regimen switch (HIV RNA > 5000 copies/mL)						
CIF only	18.2 (12.7 to 24.9) [31/170]	59.0 (47.3 to 70.0) [46/78]	49.2 (36.4 to 62.1) [31/63]	24.9 (18.8 to 31.7) [46/185]	0.4	1.4
Targeted VL	81.8 (75.1 to 87.3) [139/170]	41.0 (30.0 to 52.7) [32/78]	75.1 (68.3 to 81.2) [139/185]	50.8 (37.9 to 63.6) [32/63]	1.4	0.4
Compared with reference standard (HIV RNA > 1000 copies/mL)						
CIF only	16.9 (11.9 to 22.9) [33/195]	43.4 (29.8 to 57.7) [23/53]	52.4 (39.4 to 65.1) [33/63]	12.4 (8.0 to 18.1) [23/185]	0.3	1.9
Targeted VL	83.1 (77.1 to 88.1) [162/195]	56.6 (42.3 to 70.2) [30/53]	87.6 (81.9 to 92.0) [162/185]	47.6 (34.9 to 60.6) [30/63]	1.9	0.3
Compared with stricter definition for virological failure (HIV RNA >400 copies/mL)						
CIF only	16.7 (11.9 to 22.6) [34/203]	35.6 (21.9 to 51.2) [16/45]	54.0 (40.9 to 66.7) [34/63]	8.6 (5.0 to 13.7) [16/185]	0.3	2.3
Targeted VL	83.3 (77.4 to 88.1) [169/203]	64.4 (48.8 to 78.1) [29/45]	91.4 (86.3 to 95.0) [169/185]	46.0 (33.4 to 59.1) [29/63]	2.3	0.3

*CIF only: CIF diagnosis based on clinical (WHO clinical staging) and/or immunological (CD4 cell count) parameters. †Targeted VL: targeted viral load testing used by treating clinician in decision to switch to second-line treatment. ‡Numbers in parentheses refer to the 95% confidence interval. §Numbers in square brackets refer to the numerator and denominator from which the relevant percentages are calculated. PLR, positive likelihood ratio; NPV, negative predictive value; NLR, negative likelihood ratio; PPV, positive predictive value.

in 53 (21.2%) patients: 30 (46.9%) in the CIF only group versus 23 (12.4%) in the targeted VL group ($P < 0.001$). The CIF only criteria had a sensitivity of 16.9%, specificity 43.3%, and PPV of 52.4%. Applying a more stringent HIV RNA cut-off of <400 copies per milliliter (table 2), unnecessary switches occurred in 45 (18.1%) patients: 29 (46.0%) in the CIF only group versus 16 (8.6%) the targeted VL group ($P < 0.001$).

Genotypic analysis

Of the 195 specimens with HIV-1 RNA >1000 copies per milliliter, 183 (93.8%) valid genotypic test results were available (Fig. 1). The HIV-1 subtype distribution was C (n = 82, 44.8%), A (n = 45, 24.6%), D (n = 28, 15.3%), G (n = 14, 7.7%), CRF02_AG (n = 12, 6.6%), and other (n = 2, 1.2%). Resistance profiles are listed in table 3. At least 1 drug resistance

Table 3. Resistance patterns, by type of identification of failure

Resistance pattern	Overall (n=183)	CIF only (n=32)	Targeted VL (n=151)	*P*
Any resistance mutation	161 (88.0)	25 (78.1)	136 (90.1)	0.059
NRTI and NNRTI mutations	149 (81.4)	23 (71.8)	126 (83.4)	0.126
NRTI, NNRTI, and PI mutations	3 (1.6)	1 (3.1)	2 (1.3)	0.440
Any NRTI mutation	155 (84.7)	24 (75.0)	131 (86.8)	0.093
M184V/I	150 (82.0)	23 (71.9)	127 (84.1)	0.102
M184V/I only	0			n/a
M184V/I + TAM	97 (53.0)	18 (56.3)	79 (52.3)	0.686
TAM-containing virus*	100 (54.6)	19 (59.4)	81 (53.6)	0.554
≥2 TAMs	69 (37.7)	14 (43.8)	55 (36.4)	0.437
≥3 TAMs	44 (24.0)	12 (37.5)	32 (21.2)	0.050
K65R	13 (7.1)	3 (9.4)	10 (6.6)	0.404
K65R + TAM	3 (1.5)	1 (3.0)	2 (1.2)	0.428
K70E	6 (3.3)	0	6 (4.0)	n/a
Q151M	6 (3.3)	2 (6.3)	4 (2.7)	0.282
Q151M + TAM	3 (1.5)	0	3 (1.9)	n/a
Q151M + K65R	3 (1.5)	2 (6.0)	1 (0.6)	0.075
NRTI cross-resistance‡	87 (48)	17 (53.1)	70 (46.4)	0.486
Any NNRTI mutation	155 (84.7)	24 (75.0)	131 (86.8)	0.093
K103N	73 (39.9)	6 (18.8)	67 (44.4)	0.007
V106A/M	17 (9.3)	0	17 (11.3)	n/a
Y181C/V	57 (31.1)	13 (40.6)	44 (29.1)	0.202
G190A/S	50 (27.3)	11 (34.4)	39 (25.8)	0.324
Any PI mutation	6 (3.3)	2 (6.3)	4 (2.7)	0.282

Data are n (%) of patients. ‡NRTI cross-resistance, defined as ≥2 TAMs or Q151M or K65R/K70E. *Frequencies of individual TAMs: M41L, 40 (21.9); D67N, 46 (25.1); K70R, 39 (20.6); L210W, 17 (9.3); T215Y/F, 64 (34.9); K219Q/E, 37 (20.2). n/a, not applicable.

mutation was present in 161 of 183 (88.0%) specimens; 149 (81.4%) harbored dual-class and 3 (1.6%) triple-class resistance. Wild-type virus was detected in 7 (21.9%) patients in the CIF only group and 15 (9.9%) patients in the targeted VL group ($P = 0.059$). The most frequently observed mutation was the M184V/I (n = 150, 82.0%), followed by TAMs (n = 100, 54.6%). At least 2 TAMs were present in 69 (37.7%) specimens and 3 or more in 44 (24%). Both TAM pathways 1 and 2 were observed; the M41L was present in 40 (21.9%) specimens and the D67N in 46 (25.1%) specimens. The M184V/I mutation was combined with TAMs in 97 (53.0%) sequences. The K65R and K70E mutations were observed in 13 (7.1%) and 6 (3.3%) specimens, respectively. Three (1.5%) specimens harbored both the K65R and TAM(s). Six (3.3%) specimens harbored the Q151M complex, of which 3 with TAM(s) and 3 with the K65R. Overall, NRTI cross-resistance mutations were observed in 87 (48.0%) specimens. NRTI-associated mutational patterns did not differ by type of identification of failure (CIF only vs. targeted VL). The most frequent mutation conferring resistance to NNRTIs was the K103N (n = 73, 39.9%), followed by the Y181C/V (n = 57, 31.1%) and the G190A/S (n = 50, 27.3%). Major PI mutations occurred in 6 (3.3%) patients.

Factors associated with accumulation of drug resistance mutations

Table 4 summarizes factors associated with the presence of ≥2 TAMs and NRTI cross-resistance. In univariate analysis, the presence of ≥2 TAMs was associated with HIV-1 subtype, the total duration of previous ARV exposure, a history of ZDV use in failing and/or previous regimens and ≥2 different NRTIs. In multivariate analysis, the association persisted for ZDV use (OR: 3.49, 95% CI: 1.46 to 8.32, $P = 0.005$) and the duration of previous ARV exposure (OR for >24 months 2.90, 95% CI: 1.05 to 8.00, $P = 0.040$; OR for >36 months 4.47, 95% CI: 1.89 to 10.59, $P = 0.001$). The presence of multiple TAMs was not associated with sex, age, type of failure identification, WHO clinical stage, CD4 count, HIV RNA load, or TDF use.

The presence of NRTI cross-resistance was univariately associated with HIV RNA load, duration of ARV exposure, ≥2 different NRTIs, ZDV and TDF use, and CD4 count. Multivariate analysis showed that the risk of NRTI cross-resistance was significantly increased for longer duration of previous ARV exposure (OR: for >36 months 3.95, 95% CI: 1.58 to 9.85, $P = 0.003$), ZDV use (OR: 2.66, 95% CI: 1.12 to 6.28, $P = 0.026$) and TDF use (OR: 5.00, 95% CI: 1.67 to 14.94, $P = 0.004$). The association with HIV RNA load was close to significance (OR: 1.57, 95% CI: 1.00 to 2.47, $P = 0.052$). NRTI cross-resistance was not associated with sex, age, type of failure identification, WHO clinical stage, or HIV-1 subtype. In multivariate analysis, the K65R mutation was associated with TDF use (OR: 14.33, 95% CI: 2.92 to 70.31, $P = 0.001$) and HIV RNA load (OR: 2.27, 95% CI: 1.02 to 5.08, $P = 0.045$), but not with D4T or HIV-1 subtype.

Table 4. Factors associated with the accumulation of drug resistance mutations among patients failing first-line ART

	≥2 TAMs		NRTI cross-resistance	
	Univariate	**Multivariate**	**Univariate**	**Multivariate**
Sex				
Female	1.0	—	1.0	—
Male	1.17 (0.64 to 2.13)	—	1.16 (0.65 to 2.08)	—
Age (yrs)	1.02 (0.99 to 1.05)	—	1.01 (0.97 to 1.04)	—
Type of failure identification				
Targeted VL	1.0	—	1.0	—
CIF only	1.36 (0.63 to 2.94)	—	1.31 (0.61 to 2.82)	—
WHO clinical stage				
Stage I/II	1.0	—	1.0	—
Stage III/IV	0.99 (0.54 to 1.80)	—	1.23 (0.69 to 2.21)	—
CD4 count (cells/mm3)*	0.80 (0.61 to 1.06)	—	0.69 (0.53 to 0.92)	—
HIV RNA (log_{10})	1.09 (0.73 to 1.60)	—	1.60 (1.08 to 2.38)	1.57 (1.00 to 2.47)
HIV-1 subtype				
C	1.0	—	1.0	—
A	1.72 (0.81 to 3.65)	—	1.86 (0.89 to 3.87)	—
D	2.49 (1.03 to 5.97)	—	2.29 (0.95 to 5.52)	—
Other†	0.86 (0.34 to 2.21)	—	1.11 (0.47 to 2.66)	—
Duration of ARV use				
<24 months	1.0	1.0	1.0	1.0
24–36 months	2.97 (1.11 to 7.94)	2.90 (1.05 to 8.00)	1.6 (0.66 to 3.85)	1.99 (0.71 to 5.52)
>36 months	4.47 (1.95 to 10.23)	4.47 (1.89 to 10.59)	2.6 (1.27 to 5.31)	3.95 (1.58 to 9.85)
Thymidine analogue				
D4T	1.0	1.0	1.0	1.0
ZDV	3.02 (1.34 to 6.80)	3.49 (1.46 to 8.32)	2.43 (1.12 to 5.24)	2.66 (1.12 to 6.28)
TDF use				
No	1.0	—	1.0	1.0
Yes	0.75 (0.30 to 1.84)	—	4.19 (1.59 to 11.06)	5.00 (1.67 to 14.94)
Number of NRTIs used‡				
1	1.0	—	1.0	1.0
2 or more	2.71 (1.46 to 5.01)	—	2.83 (1.55 to 5.18)	1.09 (0.45 to 2.69)

Data are given as odds ratio (95% confidence interval). Outcomes include the emergence of ≥2 TAMs or NRTI cross-resistance, defined as: ≥ 2 TAMs or the Q151M complex or TDF-associated mutations (K65R/K70E). *OR for 100-cell increase of CD4 count. †Includes subtype G and circulating recombinant forms. ‡Excluding lamivudine or emtricitabine.

The NNRTI mutational profiles differed for patients failing efavirenz (EFV) versus nevirapine (NVP)-containing regimens. In univariate analysis, the use of EFV was associated with the K103N and V106A/M mutations, and NVP was associated with the Y181C/V. Multivariate analysis showed that EFV remained associated with the V106A/M (OR: 11.05, 95% CI: 2.39 to 50.98, $P = 0.002$) and NVP with the Y181C/V (OR: 28.5, 95% CI: 6.5 to 123.8, $P < 0.001$).

DISCUSSION

This multicenter observational study in 6 African countries investigated consequences of the common practice of using clinico-immunological criteria to detect ART failure and subsequently guide regimen switches. Without access to viral load testing, 47% of patients was switched to second-line ART inappropriately, at an HIV RNA below 1000 copies per milliliter. Targeted viral load testing to confirm treatment failure reduced unnecessary switches nearly 4-fold. A high frequency (88%) of clinically significant mutations was observed after first-line failure, suggesting late failure detection. Mutations associated with cross-resistance to NRTIs were observed in 48% of patients, comprising multiple TAMs (37.7%), K65R (7.1%), K70E (3.3%), or Q151M (3.3%). Accumulation of TAMs and NRTI cross-resistance were both associated with the duration of previous ARV exposure and ZDV use, and NRTI cross-resistance was additionally associated with TDF use.

Our data provide evidence that the use of clinico-immunological criteria to guide regimen switching has far-reaching public health implications. Treatment switches in patients who do not experience virological failure will result in mounting treatment costs and exhaust drug options. Additional concerns are the potential drug toxicity associated with second-line drugs and the costs of monitoring for adverse effects. This has particularly serious consequences in African countries where access to second-line regimens is limited. Moreover, continuation of treatment regimens in patients with virological failure will compromise their immunological and clinical status and, because of ongoing viral replication, result in the selection of viruses with accumulated resistance mutations. The effectiveness of future regimens, especially of those including NRTI backbones, is likely to be impaired.

An important strength of the study was the inclusion of a large international sample of patients diagnosed with treatment failure at different types of clinics, representative of the current clinical practice in many African ART programs. A limitation related to the cross-sectional design is that the prevalent cases identified in this study may not be rep-

resentative of all incident cases with virological and/or CIF. For example, individuals who died before the study or in whom failure was not yet diagnosed could not be included. Therefore, care should be taken when extrapolating the study results. Unfortunately, information about the duration of treatment failure or possible previous periods of clinical immunological of virological failure was not available. Additionally, historical information about prior ARV use was collected retrospectively and might have been incomplete due to limitations in recall.

Detection of treatment failure

The poor diagnostic performance of clinical and immunological criteria for the identification of virological failure observed in this study confirms previous reports 4-7]. The PPV of CIF only criteria was somewhat higher in this study, which is explained by the fact that the PPV is dependent on the prevalence of treatment failure in the sampled population [15], which was considerably higher in our study sample that consisted of patients who were switched to second-line ART, compared with the general population of patients receiving first-line ART. Our study underscores the importance of targeted viral load testing to maximize the clinical benefits of first-line regimens and prevent unnecessary switches to expensive second-line ART. This approach is in line with new WHO guidelines [3] and also corroborates findings of a recent study in India in which targeted viral load testing prevented unnecessary switch, at an HIV RNA <400 copies per milliliter, in nearly 25% of patients [16]. Taking into account that the cost of second-line regimens is currently up to 10 times higher than the cost of first-line ART, and that in our study approximately half of individuals with CIF only were truly failing, confirmation of failure by a viral load test before switching is likely to be cost saving. As simplified and more affordable methods of HIV RNA determination are being developed, the cost-effectiveness of this strategy is expected to increase further.

Despite access to local HIV RNA testing, 12% of patients were switched to second-line ART at a reference HIV RNA below 1000 copies per milliliter. The local HIV RNA had higher average values than the reference HIV RNA. This could be explained by differences between HIV RNA assays [17, 18] and factors-related specimen storage and shipment which may account for the variation in results. Another reason for the disagreement might be due to reinforcement of drug adherence although awaiting the requested test results, resulting in a suppression of HIV RNA by the continued first-line ART regimen. A previous study has shown that resuppression can occur in up to 41% of patients with viremia on NNRTI-based regimens [19]. Unfortunately, information on adherence or the time-lag between local HIV RNA testing and switch to second-line ART was not available in our study.

Drug resistance patterns and predictors

The most frequently observed mutation in our study, the M184V, causes resistance to lamivudine and emtricitabine, but delays the emergence of mutations associated with ZDV and D4T [20, 21]. Despite this increased susceptibility, we found high frequencies of multiple TAMs and 53% of patients harbored TAMs in combination with the M184V/I mutation. The rate of TAMs found in our study was higher than has previously been reported in similar population [22]. Furthermore, the considerable frequencies of K65R, K70E, and Q151M mutations observed are also likely to reflect a longer duration of treatment failure. Particularly, the Q151M complex usually requires a lengthy period of time to develop and confers broad NRTI cross-resistance [23]. K65R selection has been associated with higher levels of viral replication and lower CD4 cell counts, related to longer duration of virological failure [24]. TDF, and less frequently D4T, select for K65R [25-27], and in vitro studies have demonstrated its preferential acquisition in subtype C [28, 29]. Our analyses indeed showed that K65R was significantly associated with HIV RNA load and TDF use but did not demonstrate an association with D4T use or HIV-1 subtype.

The presence of ≥2 TAMs was associated with ZDV but not with D4T use. This is in accordance with findings of a previous study from South Africa [26], but differs from previous reports in subtype B [30]. Apart from genetic diversity, a possible explanation might be that short-term drug intolerance or toxicity is more frequently reported in relation to ZDV use, possibly leading to suboptimal adherence and prompting drug substitutions more often than in patients starting a D4T-based regimen [31]. Both TAM pathways 1 and 2 were observed, with the latter being slightly more common, which is congruent with previous reports from South Africa [26, 32].

Patterns of NRTI-associated mutations did not differ significantly according to the type of failure identification used. This is due to the fact that patients in the targeted VL group were also diagnosed with CIF and, consequently, failure was detected at a late stage. Indirect evidence for the accumulation of mutations due to late failure detection is provided by the significant associations of ≥2 TAMs and NRTI cross-resistance with the duration of previous ARV exposure. Also, HIV RNA load was marginally associated with NRTI cross-resistance.

NNRTI mutational patterns were in agreement with previous investigations [26, 33] and varied depending on prior EFV or NVP use. EFV was associated with the V106A/M mutation, whereas NVP selected for the Y181C/V mutation. Because only the Y181C/V reduces susceptibility to etravirine [34], this finding is relevant if etravirine would be considered for future salvage therapy and would encourage the use of EFV over NVP as part of first-line regimens.

Implications for clinical management

The high frequency of mutations conferring NRTI cross-resistance detected in our study may have consequences for the effectiveness of the currently available second-line regimens in African countries. Although favorable initial response to second-line ART have been described in resource-limited settings despite resistance at time of regimen switch [35-38], the development of NRTI cross-resistance will result in limited or no additional effect of the NRTI backbone, and standard second-line regimens will therefore primarily offer the benefit of the boosted-PI. Patients will thus receive functional monotherapy as second-line, which lowers the barrier for selection of PI resistance [39]. As a conditional recommendation, the WHO now advocates to perform routine viral load testing to detect virological failure early [3]. This strategy can trigger adherence interventions or changes in therapy that will limit ongoing viral replications, reducing the risk of accumulation of resistance mutations, and protect susceptibility to second-line and subsequent therapies.

In conclusion, this study demonstrated that, in the absence of viral load monitoring, unnecessary regimen switches are common, resulting in increased treatment costs and loss of drug options. Additionally, late detection of treatment failure resulted in high frequencies of accumulated mutations conferring broad cross-resistance to NRTIs, which may impair the effectiveness of second-line regimens. Future studies should determine the optimal frequency of routine virological monitoring and examine the clinical benefits of early failure detection and timely switching. The development of more affordable point-of-care viral load assays is a public health priority for resource-limited settings.

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AFRIKA

Chapter 11

Second-line antiretroviral treatment successfully resuppresses drug-resistant HIV-1 after first-line failure: prospective cohort in sub-Saharan Africa

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ABSTRACT

Little is known about the effect of human immunodeficiency virus type 1 (HIV-1) resistance mutations present at time of regimen switch on the response to second-line antiretroviral therapy in Africa. In adults who switched to boosted protease inhibitor-based regimens after first-line failure, HIV-RNA and genotypic resistance testing was performed at switch and after 12 months. Factors associated with treatment failure were assessed using logistic regression. Of 243 participants, 53% were predicted to receive partially active second-line regimens due to drug resistance. The risk of treatment failure was, however, not increased in these participants. In this African cohort, boosted protease inhibitors successfully resuppressed drug-resistant HIV after first-line failure.

INTRODUCTION

With more human immunodeficiency virus type 1 (HIV-1) infected people receiving antiretroviral therapy (ART) in low-resource settings, treatment failure and the need to switch to second-line regimens is likely to increase. Reported regimen switching rates have been lower than expected [1, 2] due in part to actual rates of treatment success, but also because of restricted access to virological monitoring and second-line regimens. The absence of virological monitoring is associated with delayed switching and consequent accumulation of resistance mutations to nucleoside reverse transcriptase inhibitors (NRTIs) [3, 4]. Lack of access to genotypic resistance testing further complicates the selection of optimal second-line regimens. Few data exist on the impact of resistance mutations selected for by the first-line regimen on the response to empirically prescribed second-line ART in resource-poor settings [5].

This study investigated the impact of acquired drug HIV-1 drug resistance mutations present at time of regimen switch on the response to second-line ART, within the PharmAccess African Studies to Evaluate Resistance Monitoring (PASER-M) cohort in 6 sub-Saharan African countries.

METHODS

Study design and population

PASER-M is a prospective cohort of adults infected with HIV-1 who receive ART at 13 clinical sites in Kenya, Nigeria, South Africa, Uganda, Zambia and Zimbabwe. Cohort and site characteristics have been profiled elsewhere [6]. Participants were consecutively enrolled during a median site-specific enrolment period of 12 months between March 2007 and September 2009. The present analysis included participants who were switched to second-line ART after first-line failure had been diagnosed using clinical, immunological and/or virological failure criteria [7]. We excluded participants who had received protease inhibitors (PIs) prior to switch, or who were pregnant at study screening. Human immunodeficiency virus type 2 (HIV-2) co-infection was ruled out using an HIV-2 specific antibody test in endemic countries (i.e. Nigeria). The study protocol was approved by the appropriate national research ethics committees and the Academic Medical Center of the University of Amsterdam in The Netherlands. Participants provided written informed consent at enrolment.

Procedures

Participants were treated and followed-up as per local standard of care, generally in accordance with 2006 World Health Organization guidelines [7]. Medical staff at each site completed case-report forms at 3-month intervals, which were entered into an online database. A data monitoring team reviewed study data. Drug adherence was assessed at each follow-up visit by 2 measures of self-reported adherence. For 3-day self-reports, the number of follow-up visits at which the patients reported to have missed any pills during the previous 3 days were counted. For the 30-day visual analogue scale, the number of pills taken at all follow-up visits was averaged and classified as <95% or >95%.

Blood collection was performed prior to regimen switch and after 12 months of follow-up (window, 11-15 months). Plasma specimens were batch-shipped to either of 2 reference laboratories in South Africa and Uganda for HIV-RNA determination, and genotypic resistance testing if HIV-RNA was >1000 copies/mL, as described elsewhere [3]. Drug resistance mutations (DRMs) were scored according to the 2010 International Antiviral Society-USA list [8]. HIV-1 subtypes were determined using the STAR algorithm and confirmed with the REGA subtyping algorithm (version 2.0). All sequences have been deposited in GenBank.

Drug resistance profiles at time of switch have been reported elsewhere [3]. Drug-susceptibility was scored using the Stanford algorithm (version 6.0.9) [9] in participants who harbored at least 1 DRM. Participants with Stanford levels 3 (low-level resistance), 4 (intermediate resistance), or 5 (high-level resistance) to at least one of their prescribed drugs were considered to have received partially-active ART. Participants with Stanford levels 1 (susceptible) or 2 (potential low-level resistance) to all prescribed drugs were considered to have received fully-active ART. Participants who had HIV-RNA <1000 copies/mL, or HIV-RNA >1000 copies/mL without DRMs were also considered to have received fully-active ART. GenBank sequence accession numbers JN132214-JN132396, JN393292-JN393306.

Among participants still in follow-up after 12 months, virological failure was defined as an HIV-RNA of ≥400 copies/mL. Immunological failure was defined according to WHO guidelines as a decrease in CD4 cell count to the value before regimen switch, a decline of at least 50% from highest measurement on treatment, or a persistent CD4 cell count <100 cells/mm^3. [7]. Clinical failure was defined as the presence of a new WHO clinical stage 4 event or new diagnosis of pulmonary tuberculosis.

Statistical analysis

Group comparisons for categorical data were done using chi-square or Fisher exact test, and for continuous data using 1-way analysis of variance or Kruskal-Wallis test. Logistic regression with robust standard errors, accounting for clustering of observations within sites, was used to identify risk factors for the 2 outcomes virological failure and any type of failure. Attrition was additionally considered as virological failure. Any type of failure was defined as virological, immunological or clinical failure, or attrition. Explanatory variables at time of switch to second-line ART included the activity of the second-line regimen, age, sex, WHO clinical stage, CD4 cell count, and HIV-RNA load. Prospective parameters included any single-drug substitutions, 30-day and 3-day adherence. In addition to activity of the second-line regimen, age and sex, all variables univariately associated ($p<0.05$) with the outcome were stepwise entered into the multivariate model. Results were expressed as odds ratios (ORs) with 95% confidence intervals (CI) and two-sided p-values, with $p<0.05$ regarded statistically significant. All statistical analyses were performed using Stata version 10 (StataCorp LP, TX, USA).

RESULTS

Study population

We enrolled 243 participants who switched to a second-line PI-based regimen after first-line ART failure. HIV-RNA and genotypic test results were available for 232 participants (95.5%), for whom the predicted activity of the second-line regimen could be determined. ART was predicted to be fully active for 104 participants (44.8%), comprising 50 participants (48.1%) with HIV-RNA <1000 copies/mL, 22 participants (21.2%) with HIV-RNA >1000 copies/mL and wild-type virus, and 32 participants (30.8%) with HIV-RNA >1000 copies/mL and drug-resistant virus at Stanford levels 1 or 2. ART was predicted to be partially active for 128 participants (55.2%), harboring drug-resistant virus with reduced predicted susceptibility to at least 1 prescribed drug. Of these, 60 (46.9%) received <2 active drugs. Among participants with drug-resistant virus, NRTI-associated DRMs were observed in 154 (96.3%), NNRTI-associated DRMs in 153 (95.6%) and dual-class resistance in 147 (91.9%).

At second-line start, WHO clinical stage 4 was diagnosed in 12 participants (11.5%) predicted to receive fully active regimens and in 25 participants (19.5%) with partially active regimens ($p=0.014$, table 1). For the 2 groups, median CD4 cell count was 147 and 104 cells/mm^3 ($p=0.001$) and median HIV-RNA load was 3.2 and 4.7 $\log_{10}$ copies/ml ($p<0.001$), respectively. The median duration of first-line ART was 26.7 months; participants predicted to receive fully active regimens had shorter duration of previous ART

Table 1. Participant characteristics at second-line start, stratified by regimen activity

	Overall (n=232)	Fully active (n=104)	Partially active (n=128)	*p*[a]
Women	116 (50)	56 (53.9)	60 (46.0)	0.291
Median age, yrs	38.3 [34.1-45.1]	38.5 [34.4-44.5]	38.2 [33.8-45.9]	0.972
WHO clinical stage				0.014
1 or 2	119 (51.3)	57 (24.6)	62 (26.7)	
3	76 (32.8)	35 (33.7)	41 (32.0)	
4	37 (16.0)	12 (11.5)	25 (19.5)	
Median CD4 cell count (cells/mm^3)[b]	126 [66-205]	147 [92-230]	104 [51-185]	0.001
Median HIV-RNA (log_{10} c/ml)	4.2 [3.3-5.0]	3.2 [1.6-4.2]	4.7 [4.1-5.2]	<0.001
Median duration of first-line regimen (months)[c]	26.7 [15.2-43.7]	22.8 [13.8-39.5]	30.1 [16.5-48.7]	0.024
ART regimen at failure[d]				0.001
ZDV, 3TC, NNRTI	102 (44.0)	39 (37.5)	63 (49.2)	
D4T, 3TC, NNRTI	95 (41.0)	59 (56.7)	36 (28.1)	
TDF, FTC, NNRTI	23 (9.9)	3 (2.9)	20 (15.6)	
NNRTI-based, other	5 (2.2)	2 (1.9)	3 (2.3)	
Triple NRTI	4 (1.7)	0	4 (3.1)	
Criteria used for switch[e]				0.006
Clinico-immunological only	60 (25.9)	36 (34.6)	24 (18.8)	
Targeted viral load testing	172 (74.1)	68 (65.4)	104 (81.3)	
Country of origin				0.002
Uganda	73 (31.5)	21 (20.2)	52 (40.6)	
South Africa	59 (25.4)	34 (32.7)	25 (19.5)	
Kenya	36 (15.5)	22 (21.2)	14 (10.9)	
Nigeria	30 (12.9)	16 (15.4)	14 (10.9)	
Zambia	24 (10.3)	9 (8.7)	15 (11.7)	
Zimbabwe	10 (4.3)	2 (1.9)	8 (6.3)	

Percentages are shown in parentheses and interquartile ranges in square brackets. [a] Categorical data were compared using the chi-square test; continuous were compared using the Wilcoxon rank-sum test. [b] 230 observations. [c] 227 observations. [d] 229 observations. [e] Clinico-immunological criteria for switch was defined as WHO clinical staging or CD4 counts only. Targeted viral load testing was defined as ART failure based on clinical and immunological information, confirmed with an HIV-RNA test. ART, antiretroviral treatment; ZDV, zidovudine; 3TC, lamivudine; NNRTI, non-nucleoside reverse transcriptase inhibitor; D4T, stavudine; TDF, tenofovir; FTC, emtricitabine.

than participants with partially active ART (22.8 versus 30.1 months, p=0.024). Subtype C was most commonly identified (n=77, 42.3%), followed by A (n=49, 26.9%), D (n=26, 14.3%), G (n=14, 7.7%), and circulating recombinant forms (CRFs) (n=16, 8.8%).

Response to second-line ART

After 12 months of follow-up, 208 participants were still on second-line ART and 201 had available HIV-RNA results. Of these, 28 participants (13.9%) experienced virologi-

cal failure. Eleven of 80 participants (13.8%) predicted to receive fully active regimens and 17 of 112 (15.2%) of those with partially active ART experienced virological failure (p=0.782). Of participants predicted to receive <2 active drugs, 7 of 51 (13.7%) experienced virological failure.

Of participants with an HIV-RNA load >1000 copies/mL after 12 months of follow-up, 15 of 20 (75%) had a valid genotype. Nine (60%) harboured ≥1 major DRMs. Detected mutations were associated with NRTIs (n=7, 46.7%), NNRTIs (n=8, 53.3%) and PIs (n=1, 6.7%). There were no significant differences in DRM patterns between participants predicted to receive a fully versus partially active second-line regimens.

CD4 cell counts were available for 173 of 208 participants still in follow-up. The median CD4 cell count gain was 146.5 cells/mm^3. Immunological failure was diagnosed in 21 participants (12.1%). Information on WHO clinical stage was available for all participants, and 13 (6.3%) experienced clinical failure. Clinical and immunological failure rates did not differ significantly for participants predicted to receive fully or partially active second-line regimens.

An HIV-related cause of death was recorded in 11 participants (4.5%). Other participants not retained in care were lost to follow-up (n=19, 7.8%) or transferred out (n=4, 1.6%). One person switched to a third-line regimen due to alleged virological failure before the 12 month visit. The attrition rates did not differ significantly for participants predicted to receive fully or partially active second-line regimens.

Risk factors for failure of second-line ART

In multivariate analyses, the predicted activity of the second-line regimen was not significantly associated with virological failure or any type of failure (table 2). The risk of virological failure was increased for patients with 30-day adherence <95%, and reduced for increasing age. The risk of any type of failure was increased for patients with 30-day adherence <95% and for those in clinical stage 4 at second-line start.

DISCUSSION

In this assessment of routine ART programs in sub-Saharan Africa, the risk of second-line ART failure was not increased in participants who harbored virus with predicted reduced susceptibility to at least 1 prescribed second-line drug, compared to those who received ART that was predicted to be fully active. After 12 months of second-line ART, 14% of participants experienced virological failure, 5% died and 8% were lost to follow up. Our

Table 2. Risk factors for second-line failure

	Virological failure[a]				Any type of failure[b]			
	Events (%)	**Univariate**	**Multivariate**	**p**	**Events (%)**	**Univariate**	**Multivariate model**	**p**
Activity of second-line regimen[c]								
Fully-active	31 (29.8)	1.0	1.0		44 (42.3)	1.0	1.0	
Partially-active	30 (23.4)	0.72 (0.38-1.36)	0.80 (0.33-1.91)	0.610	39 (30.5)	0.60 (0.37-0.99)	0.53 (0.24-1.17)	0.115
Sex								
Female	28 (23.0)	1.0	1.0		40 (32.8)	1.0	1.0	
Male	35 (28.9)	1.37 (0.74-2.51)	1.76 (0.65-4.72)	0.263	47 (38.8)	1.30 (0.73-2.32)	1.55 (0.57-4.27)	0.393
Age (years)		0.98 (0.94-1.01)	0.94 (0.90-0.98)	0.006		0.99 (0.95-1.02)	0.96 (0.92-1.00)	0.083
Clinical stage at second-line start								
1-3	48 (23.5)	1.0	1.0		62 (30.4)	1.0	1.0	
4	15 (38.5)	2.03 (0.78-5.30)	2.44 (0.97-6.17)	0.059	25 (64.1)	4.09 (1.33-12.57)	5.25 (1.55-17.74)	0.008
CD4 count at second-line start								
≥100 cells/mm^3	35 (24.7)	1.0			53 (37.3)	1.0		
<100 cells/mm^3	27 (27.3)	1.15 (0.63-2.10)			33 (33.3)	0.84 (0.53-1.33)		
VL at second-line start (log_{10} c/ml)		1.19 (0.97-1.45)				1.07 (0.87-1.31)		
Substitutions								
None	58 (13.2)	1.0	1.0		81 (35.4)	1.0		
Yes, ≥1	5 (33.3)	1.64 (0.78-3.46)	2.59 (0.95-7.08)	0.063	6 (42.9)	1.37 (0.64-2.95)		
3-day adherence[d]								
No pills missed	32 (16.6)	1.0			50 (25.9)	1.0		
Pills missed at ≥1 visit	12 (38.7)	3.18 (1.05-9.64)			18 (58.1)	3.96 (1.29-12.18)		
Average 30-day adherence (%)[e]								
≥95%	35 (17.2)	1.0	1.0		55 (27.0)	1.0	1.0	
<95%	7 (38.9)	3.07 (1.51-6.25)	2.90 (1.12-7.54)	0.029	11 (61.1)	4.26 (1.80-10.08)	4.08 (1.45-11.46)	0.008

Multivariate logistic regression with robust standard errors. Data are given as odds ratio (95% confidence interval). VL, viral load.

[a] Virological failure defined as HIV-RNA ≥ 400 copies/mL (n=28). Participants that died (n=11), were lost to follow-up (n=19), transferred out (n=4) or switched to third-line ART (n=1) were additionally considered as virological failure; [b] Any type of failure defined as clinical, immunological or virological failure (n=63). Participants who were lost to follow-up, transferred out or switched to third-line ART were additionally considered as any type of failure; [c] Activity of second-line regimen for participants harboring DRMs based on the Stanford drug-susceptibility algorithm (version 6.0.9); [d] The number of follow-up visits at which the participant reported to have missed any pills during three days prior; [e] The average percentage of pills taken during 30 days prior to all follow-up visits.

study demonstrates that empirically prescribed PI-based regimens can successfully resuppress HIV, even in the absence of a fully active NRTI backbone. This corroborates findings of a Malawian study among patients with extensive NRTI resistance at the time of switch [5]. Our study adds important insights to the limited available data on the effectiveness of second-line ART in resource-limited settings [10, 11].

The good immunological and virological response to partially active second-line regimens, i.e., in participants who harbored NRTI resistance, is likely explained by the high potency and genetic barrier to resistance of ritonavir-boosted PIs [12]. Our data suggest potential for the use of "simplified" PI-based regimens after failure of an NNRTI-based regimen. Preliminary results from the ACTG 5230 trial, evaluating PI monotherapy after first-line treatment failure in low-resource settings, showed promising results with 87% virological suppression after 6 months [13]. By contrast, a meta-analysis and recent trial have shown that boosted PI monotherapy is inferior to standard triple ART regimens and cannot be considered an alternative to standard treatment [14, 15]. Therefore, long-term results from the ACTG 5230 and other ongoing studies, such as the EARNEST trial, are eagerly awaited.

Participants with predicted partially active second-line regimens had more advanced HIV disease, lower CD4 cell counts and higher viral loads, which is in line with the observation that prolonged ART failure leads to disease progression and drug resistance accumulation [3, 4]. Starting second-line ART after the occurrence of an AIDS-defining event increased the risk of treatment failure, indicating that regimen switch should ideally occur before the onset of severe clinical symptoms. Early detection of first-line failure can be improved by implementing routine virological monitoring, which has also been shown to avert unnecessary switches in the absence of viral breakthrough [3]. Furthermore, access to affordable second-line drugs needs to be urgently improved to enable switching to second-line ART when appropriate.

Our finding that suboptimum adherence to second-line ART was associated with an increased risk of treatment failure expands on previous knowledge [5, 10], and underscores the importance of meticulous long-term adherence. A limitation of our study is the fact that our findings apply to the first year of second-line ART. Longer term follow-up is required to determine if these early outcomes can be sustained over the following years of treatment. Secondly, there was an overrepresentation of urban sites in our cohort and therefore caution is warranted when extrapolating the results to other settings where resource constraints may be more significant.

In conclusion, our cohort study in 6 African countries showed that individuals on second-line ART achieve favorable virological outcomes after 12 months of follow-up, despite the fact that more than half were predicted to receive partially active regimens. Given that the provision of effective and safe ART requires life-long commitment, it is a global health priority to ensure access to viral load testing and to improve availability of second-line drugs.

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AFRIKA

Part III

Public health policy


AFRIKA

Chapter 12

Building capacity for the assessment of HIV drug resistance: Experiences from the PharmAccess African Studies to Evaluate Resistance Network

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ABSTRACT

The PharmAccess African Studies to Evaluate Resistance (PASER) network was established as a collaborative partnership of clinical sites, laboratories, and research groups in 6 African countries; its purpose is to build research and laboratory capacity in support of a coordinated effort to assess population-level acquired and transmitted human immunodeficiency virus type-1 drug resistance (HIVDR), thus contributing to the goals of the World Health Organization Global HIV Drug Resistance Network. PASER disseminates information to medical professionals and policy makers and conducts observational research related to HIVDR. The sustainability of the network is challenged by funding limitations, constraints in human resources, a vulnerable general health infrastructure, and high cost and complexity of molecular diagnostic testing. This report highlights experiences and challenges in the PASER network from 2006 to 2010.

INTRODUCTION

Despite enormous progress, major challenges remain to scaling up access to antiretroviral treatment (ART) for human immunodeficiency virus (HIV)–1-infected individuals in sub-Saharan Africa [1]. Under-resourced health systems result in important programmatic deficiencies, such as lack of virological monitoring, to detect treatment failure and inconsistent supply of antiretroviral drugs [2]. These deficiencies may contribute to the development of HIV drug resistance (HIVDR) during ART [3] and the subsequent transmission of drug-resistant strains to newly infected individuals [4], which has severe public health consequences in settings where treatment options are limited. Current conditions therefore advocate for the development of a global public health framework to assess and minimize the emergence of HIVDR [5].

The PharmAccess African Studies to Evaluate Resistance (PASER) network was established as a collaborative partnership of clinical sites, laboratories, and research groups in South Africa, Zambia, Zimbabwe, Uganda, Kenya, and Nigeria in 2006. PASER, jointly with its counterpart program TREAT Asia Studies to Evaluate Resistance (TASER) in Asia, constitutes a collaborative bi-regional capacity development program, which receives major financial support from the Ministry of Foreign Affairs of the Netherlands (http://www.laaser-hivaids.org). The PASER network strives to develop regional capacity for the coordinated population-level assessment of acquired and transmitted HIVDR, thereby advancing the epidemiological, clinical, and laboratory knowledge necessary for management of HIVDR in the sub-Saharan African region. PASER contributes to fulfilling the goals of the Global HIV Drug Resistance Network (HIVResNet), developed by the World Health Organization (WHO) [5]. The PASER study protocols focus on the assessment of acquired HIVDR in patients receiving first- or second-line ART (PASER Monitoring [PASER-M]) [6, 7, 8] and transmitted HIVDR in recently infected populations (PASER-Surveillance, PASER-S) [9, 10], and have been harmonized with the corresponding WHO generic protocols assessing acquired and transmitted HIVDR [11, 12], with the exception that PASER-M studies include longer patient follow-up and larger sample sizes and follow patients during both first- and second-line ART. PASER-M studies [6] have been implemented in 13 clinical sites in 6 African countries, and PASER-S studies have been conducted in Kampala [10] and Mombasa [9] (table 1). Subsequently, PASER has developed a number of projects and studies related to HIVDR. To make these studies possible, PASER enhanced the research capacity at participating sites and the HIVDR testing capacity at reference laboratories. This report highlights the experiences and challenges in the PASER network from 2006 to 2010.

Table 1. Summary of PASER achievements 2006-2010

	Target	Achieved
Capacity building		
HIVDR genotyping reference laboratories participating in TAQAS	3	3
Annual network meetings	5	5
Clinicians trained on HIVDR protocols and basic research skills	75	100
Laboratory staff trained on HIVDR protocols, GCLP, molecular diagnostics	75	86
Patient data		
HIVDR Monitoring studies		
Clinical sites / countries	15 / 7	13 / 6
Persons enrolled	3120	3007
Patient retention at 12 months of follow-up	2433	2360
HIVDR Surveillance studies		
Studies / countries	6 / 3	2 / 2
Persons enrolled	255	163

HIVDR, HIV-1 drug resistance; GCLP, Good Clinical Laboratory Practice; TAQAS, TREAT Asia Quality Assessment Scheme

NETWORK DEVELOPMENT

The PASER network is coordinated by PharmAccess Foundation, a nongovernment organization dedicated to the strengthening of health systems and improving access to quality basic healthcare in sub-Saharan Africa (http://www.pharmaccess.org), in collaboration with the Amsterdam Institute for Global Health and Development and the Department of Virology at the University Medical Center Utrecht. The creation of the network involved the selection of dedicated HIV treatment clinics, reference laboratories, and research groups in each of the African subregions. A total of 13 ART clinics and 3 laboratories in 6 countries were selected after careful assessments (figure 1). Participating clinical sites represent a variety of clinic types in terms of geography, available resources, administration (public, nongovernmental, private, and faith based), and level of experience with HIV treatment and research [6]. PASER is represented on the HIVResNet steering committee and collaborates with WHO HIVDR working groups at the national level.

LABORATORY CAPACITY

Given that very few laboratories in the region have the required organization and expertise to develop HIVDR testing capacity and the high cost of capital equipment

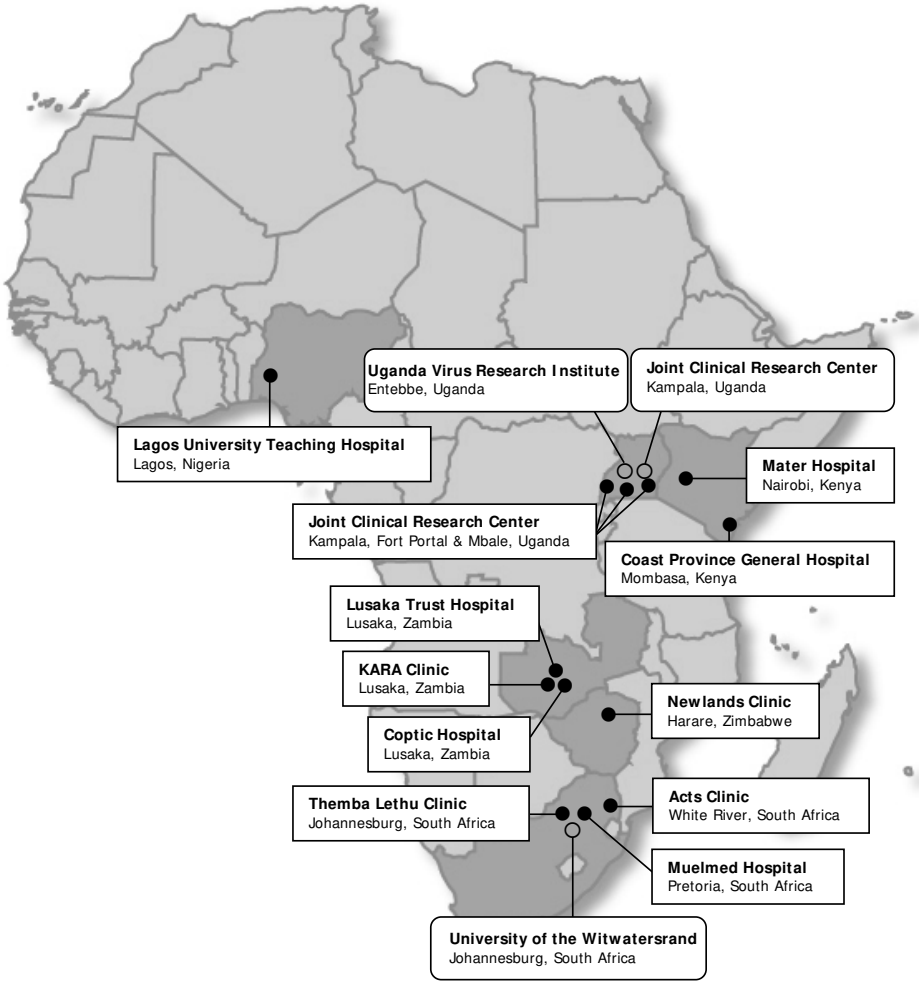

Figure 1. Geographical map of PASER sites. Closed circles represent clinical sites; open circles, reference laboratories.

for sequencing, it was decided to select and support a limited number of central reference laboratories situated in Johannesburg, Entebbe, and Kampala. Centralized laboratory testing also ensured standardization and quality assurance of the laboratory procedures. PASER provided laboratory equipment, database technology, and technical assistance, as required. Laboratory staff from all clinical sites and reference laboratories received additional training in Good Clinical Laboratory Practice and Good Molecular Diagnostics Practice, which included principles of unidirectional workflow, prevention of carryover contamination, and the principles of amplification and sequencing. Annual network meetings created a platform for laboratory staff to exchange experiences with international colleagues and interact with clinicians and study support staff. To ensure

data quality, each reference laboratory was enrolled in ≥2 external quality assurance programs for HIVDR testing. One of them is the TREAT Asia Quality Assurance Scheme (TAQAS) [13], which was set up in collaboration with a WHO-accredited regional HIVDR reference laboratory in Sydney, Australia. Before laboratories were allowed to start genotyping for PASER studies, proficiency testing was performed through TAQAS. Two reference laboratories (Johannesburg and Entebbe) have acquired WHO HIVResNet accreditation for HIVDR genotyping for public health surveillance [14], which has helped facilitate PASER studies.

HIV viral load and HIVDR genotyping was conducted on stored plasma. A consequence of centralized testing was the need for cold chain logistics across country borders. In preparation for the start of the studies, dedicated laboratory staff members received training on specimen handling and documentation according to protocol requirements, including the use of a Web-based specimen track and trace system. Sites received freezers for adequate specimen storage, if required. Funding for installing power back-up systems was not available, and, unfortunately, 1 site experienced interruptions in power supply for some time, which may have affected the quality of stored specimens. Specimen tracking data for PASER-M demonstrated 99% timely plasma separation (<12 hours), 98% timely storage at -80°C (<36 hours), and 99% receipt in adequate condition at the reference laboratory.

All generated sequences were submitted to the Web-based ViroScore Suite database (Advanced Biological Laboratories) for data storage and quality control. Due to logistic constraints, HIVDR testing was performed retrospectively and did not directly influence patient care. To address the high cost (USD 200 per test) and complexity of plasma-based genotyping [15], PASER has initiated a public-private consortium, called Affordable Resistance Test for Africa (ART-A), funded through the Netherlands-African Partnership for Capacity Development and Clinical Interventions Against Poverty-Related Diseases, which aims to develop and implement novel affordable and simple diagnostic technology for HIV viral load testing as well as the detection and interpretation of HIVDR in African clinics and laboratories (http://www.arta-africa.org).

CAPACITY FOR STUDIES TO ASSESS HIVDR

Many medical professionals in the PASER network did not have any prior research experience. Regular monitoring visits to the participating sites were conducted and were partly dedicated to teaching and training of basic research skills to site investigators, clinicians, nurses, and laboratory technicians. Annual network meetings were used to

provide skills building workshops on research methods, good clinical practice, and HIV disease management, including clinical and laboratory aspects of HIVDR: a total of 100 clinicians and 86 laboratorians received training at 5 network meetings (table 1). PASER-M has achieved 96% (n=3007) of the anticipated patient recruitment, with 82% retained in follow-up after 12 months (table 1). PASER-S studies have been successfully conducted in Kampala (78 participants) [10] and Mombasa (85 participants) (table 1). Several site investigators are actively involved in the analysis and reporting of study findings to local health policy makers and the public health community. Central clinical data collection for PASER-M was performed through the Web-based clincal database developed by PharmAccess. Despite the program's efforts to upgrade on-site information and communication technology capacity, many clinics did not have in-office personal computer workstations and reliable internet connections. Therefore, PASER-M used hard-copy data collection forms, which were subsequently transferred to the web-based database. PASER-M was initially monitored by a clinical research associate (CRA) from the Netherlands through regular site monitoring visits. As of 2008, the local study nurses at the sites in Nigeria, Kenya, and Uganda received training and mentoring in CRA monitoring. By 2010, the locally trained CRAs were capable of conducting study monitoring independently.

DISCUSSION

The PASER program has established a regional collaborative network to strengthen research and laboratory capacity for the population-level assessment of HIVDR, with effective linkages between clinical sites and reference laboratories. Key achievements of the PASER program are summarized in table 1. Building sustainable relationships and networks is important for the clinical and scientific communities within countries and regions, to facilitate the exchange of information and experience. PASER (with TASER) has brought together multidisciplinary stakeholders from academia, governments, nongovernment organizations, private sector, and civil society in Africa and Asia to draw attention to the imminent threat of HIVDR. Through the assessment of HIVDR at national and regional levels, PASER will contribute to evidence-based recommendations to inform ART guidelines and to provide feedback on the success of HIV treatment and prevention programs. The ART-A project is expected to produce simplified and affordable alternative HIVDR assessment tools, which will facilitate future HIVDR studies.

Several local challenges were faced during the development of the PASER network, such as political instability, competing interests, complexity of specimen logistics, failure to negotiate contracts, or inability to obtain ethical clearance. The study lead time was

substantially prolonged at some sites due to lengthy bureaucratic procedures in obtaining ethics approvals and permission to ship specimens abroad to the regional reference laboratories (e.g., in Ethiopia, Kenya, and Zimbabwe). The implementation of PASER-S proved challenging because of difficulties in identifying recently HIV-infected individuals even at antenatal clinics and voluntary counseling and testing sites. The sustainability of a research and surveillance network primarily depends on funding, and the current core funding for PASER will end in December 2011. Site investigators are committed to continuing the network, and efforts are ongoing to secure funding and ensure sustainability of the network. Other challenges include constraints in human resources, the need for continued training and education, the vulnerable general health infrastructure in many settings, and the urgent need for simplified and affordable diagnostic technology. The enhanced commitment of global health donors and technical agencies, including WHO, to establish and maintain surveillance networks to track the emergence and spread of HIVDR is crucial in this respect.

In conclusion, this report provides practice-based lessons from the PASER network. We believe that PASER has considerably improved the clinical research and laboratory capacity for the assessment of HIVDR in the African region. The network provides opportunities for further knowledge exchange, public health research, and health system development. For PASER to become sustainable, extended funding needs to be urgently secured, the cost and complexity of molecular laboratory testing need to be addressed, and the capacities of and collaborations between local, regional, and global institutions need to be further strengthened.

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Contributors

All authors have contributed extensively to the development and implementation of PASER. RLH wrote the first draft of the manuscript. RLH, ES, and KCES aggregated operational data. CK, CLW, WS, KCES, and TFRW critically reviewed the paper. All authors reviewed and approved the final manuscript. TFRW is the guarantor.

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AFRIKA

Chapter 13

Early warning indicators for population based monitoring of HIV drug resistance in 6 African countries

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ABSTRACT

Human immunodeficiency virus (HIV) RNA testing and HIV drug resistance (HIVDR) testing are not routinely available for therapeutic monitoring of patients receiving antiretroviral therapy (ART) in resource-limited settings. World Health Organization HIVDR early warning indicators (EWIs) assess ART site factors known to favor the emergence of HIVDR. HIV drug resistance EWI monitoring was performed within the PharmAccess African Studies to Evaluate Resistance Monitoring (PASER-M) study, comprising 13 ART sites in 6 African countries. Early warning indicator assessment in the PASER network identified vulnerable aspects of ART programs and triggered interventions aimed at minimizing HIVDR emergence. Additionally, data suggest an advantage of medication possession ratio over on-time antiretroviral drug pickup in identifying patients at risk for HIVDR development.

INTRODUCTION

Major challenges to expanded access of antiretroviral therapy (ART) for human immunodeficiency virus (HIV)–infected individuals in sub-Saharan Africa arise from a deficient, under-resourced health infrastructure. Suboptimal delivery of HIV care and treatment may accelerate the emergence of drug-resistant viruses, which could impair the sustained response to first-line ART and increase the subsequent spread to newly infected individuals [1, 2]. Because HIV drug resistance (HIVDR) testing is not routinely available in resource-limited settings, the World Health Organization (WHO), as part of its global strategy for prevention and assessment of HIVDR, recommends that ART program factors, such as prescribing practices, patient retention, and drug supply and adherence, are monitored to optimize the quality of patient care [3]. World Health Organization HIVDR early warning indicators (EWIs) make use of data that are routinely collected in patients' medical and pharmacy records [4], and feasibility of their implementation has been successfully demonstrated in sub-Saharan Africa [5, 6]. However, a formal evaluation of the EWIs for their ability to identify possible HIVDR has not been performed.

Imperfect adherence to antiretroviral (ARV) medication is associated with poor virological response [7-10] and selection of drug-resistant virus [11, 12]. Patient adherence can be approximated using pharmacy pickup information, and studies have shown that drug pickup data may be considered an alternative to CD4 cell counts in predicting virological failure [13, 14]. Another adherence measure, the medication possession ratio (MPR), defined as the amount of time an individual is in possession of ARVs divided by the time between ARV prescriptions [15], has also been shown to be associated with treatment outcomes in resource-limited settings [7, 13, 16-18].

We report the results of EWI assessment in a clinical network of 13 ART sites in 6 African countries. Additionally, we sought to evaluate the EWI on-time ARV drug pickup and the MPR as measures to determine possible HIVDR after 12 months of ART.

METHODS

Study population and design

Early warning indicators were collected as part of the PharmAccess African Studies to Evaluate Resistance Monitoring (PASER-M) study, a multicenter, prospective, observational cohort of HIV-infected adults who receive first- and second-line ART under routine circumstances at 13 clinical sites in 6 African countries (Kenya, Nigeria, South Africa, Uganda, Zambia, and Zimbabwe). The sites collaborating on the PASER-M study

comprise 6 public institutions, 3 non-governmental organizations, 2 private for-profit clinics, and 2 faith-based hospitals. Cohort and site characteristics have been previously described [19]. Exclusion criteria were pregnancy at study screening and reinitiation of first-line ART < 30 days after stopping previous ART. Any other prior ARV drug use was not an exclusion criterion. Participants were consecutively enrolled during a median site-specific enrollment period of 12 months. The appropriate institutional review boards approved the study, and all participants provided written informed consent.

EWI data abstraction

Definitions of the selected EWI followed WHO/HIVResNet guidance [4] and are summarized in table 1. Data on PASER-M participants initiating first-line ART were used to abstract the following EWIs: ART prescribing practices, patients lost to follow-up (LTFU) at 12 months, patient retention of first-line ART, and viral load (VL) suppression at 12 months. The indicators "on-time ARV drug pickups" and "ARV drug supply continuity" were retrospectively collected from medical and/or pharmacy records. Indicator 4a (on-time ARV drug pickups) was modified from the WHO indicator and assessed for whether

Table 1. Definition of selected Early Warning Indicators

Indicator	Definition
1. ART prescribing practices	Percentage of patients initiating ART at the site who are prescribed, or pick up from the pharmacy, an appropriate[a] first-line ART regimen.
2. Patients LTFU at 12 months	Percentage of patients initiating ART at the site who are LTFU[b] 12 months later.
3. Patient retention on first-line ART	Percentage of patients initiating ART at the site who are taking an appropriate[a] first-line regimen 12 months later, excluding those who transferred out.
4a. On-time ARV drug pick-ups	Percentage of patients picking up all prescribed ARV drugs on-time[c] for two consecutive drug pick-ups after initiation of ART.
4b. On-time ARV drug pick-ups for 12 months	Percentage of patients picking up all prescribed ARV drugs on-time[c] during the first 12 months of ART or until classified as dead, transferred out or stopped ART.
6. ARV drug-supply continuity	Percentage of months in a designated year in which there were no ARV drug stock-outs.
8. VL suppression at 12 months	Percentage of patients initiating ART at the site with VL <1000 copies/ml after 12 months of first-line ART, excluding those who died or transferred out.

[a] An appropriate first-line ART regimen meets one or both of the following definitions: standard regimen listed in national ART guidelines and used according to those guidelines; regimen recommended in the WHO treatment guidelines. [b] Lost to follow-up is defined as a patient who has not returned to the clinic ≤90 days after the last missed appointment or drug pick-up during the first 12 months of ART, and who was not known to have transferred out, stopped therapy without restarting, or died. [c] On or before the date the previously dispensed drugs would have run out if taken according to schedule. Table adapted from WHO HIVDR early warning indicators guidance document; ART, antiretroviral therapy; ARV, antiretroviral; VL, viral load.

patients were on time for 2 consecutive drug pickups after initiation of ART; those who had been on ART for longer periods of time were not considered.

Medication possession ratio

The MPR was assessed using pharmacy pickup information during the first 12 months of ART for all patients with the exception of those who died or transferred out. The MPR was calculated by dividing the number of days a patient was in possession of ARVs by the 365 days in the first calendar year of ART [15].

Laboratory methods

HIV RNA testing for the survey was performed on EDTA anticoagulated plasma using the NucliSens EasyQ real-time assay version 2.0 (bioMérieux, Lyon, France) or the COBAS Ampliprep/COBAS TaqMan assay (Roche, Branchburg, New Jersey) at 2 reference laboratories in South Africa and Uganda.

Classification of virological outcomes

Viral load suppression, or HIVDR prevention, was defined as HIV RNA ≤1000 copies/mL after 12 months of first-line ART [20]. The outcome "possible HIVDR" applied to patients with HIV RNA > 1000 copies/mL after 12 months but also to patients who were LTFU, who were no longer on appropriate first-line regimens (due to stopping or switching ART), or from whom no follow-up specimen was available. Patients who transferred out or died in the first year of ART were censored from analysis because these outcomes are unlikely to be the result of HIVDR [20].

Statistical Analysis

World Health Organization data abstraction tools [4] were used to capture and calculate on-time ARV drug pickups. The sensitivity, specificity, positive and negative predictive values of the EWI "on-time ARV drug pickup" and the MPR for the identification of virological outcomes (VL suppression or possible HIVDR) were calculated with 95% confidence intervals (CIs) using 2 x 2 tables. The positive and negative likelihood ratios were derived from the sensitivity and specificity. All analyses were performed using Stata software version 10 (StataCorp, College Station, Texas).

RESULTS

Study sites and population

During the recruitment period from March 2007 to September 2009, 2735 patients initiated first-line ART. Less than 5% of patients reported any previous ARV exposure (i.e.,

Table 2. Early Warning Indicators results by site

	ART prescribing practices	Patients LTFU at 12 months	Patient retention on first-line ART	On-time ARV drug pick-ups	Drug-supply continuity[a]	VL suppression at 12 months[b]
WHO target	100%	≤ 20%	≥ 70%	≥ 90%	100%	≥ 70%
Site 1	116/116 (100%)	13/116 (11.2%)	81/115 (70.4%)	-	12/12 (100%)	70/96 (72.9%)
Site 2	228/228 (100%)	10/228 (4.4%)	192/223 (86.1%)	178/228 (78.1%)	12/12 (100%)	173/206 (84.0%)
Site 3	239/239 (100%)	31/239 (13.0%)	165/227 (72.7%)	156/235[c] (66.4%)	12/12 (100%)	147/200 (73.5%)
Site 4	205/205 (100%)	20/205 (9.8%)	156/199 (78.4%)	-	na[d]	139/185 (75.1%)
Site 5	204/204 (100%)	22/204 (10.8%)	162/193 (83.9%)	-	12/12 (100%)	132/185 (71.4%)
Site 6	223/223 (100%)	34/223 (15.3%)	177/220 (80.5%)	-	12/12 (100%)	154/214 (72.0%)
Site 7	203/203 (100%)	18/203 (8.9%)	174/202 (86.1%)	-	11/12 (91.7%)	140/193 (72.5%)
Site 8	215/215 (100%)	22/215 (10.2%)	176/215 (81.9%)	-	11/12 (91.7%)	156/200 (78.0%)
Site 9	221/223 (99.1%)	17/223 (7.6%)	173/222 (77.9%)	-	9/12 (75%)	159/192 (82.8%)
Site 10	220/220 (100%)	15/220 (6.8%)	184/213 (86.4%)	-	12/12 (100%)	152/199 (78.6%)
Site 11	223/223 (100%)	15/223 (6.7%)	200/217 (92.2%)	-	12/12 (100%)	169/215 (78.6%)
Site 12	229/230 (99.6%)	7/230 (3.0%)	211/230 (91.7%)	-	12/12 (100%)	186/223 (83.4%)
Site 13	206/206 (100%)	44/206 (21.4%)	148/205 (72.2%)	-	12/12 (100%)	127/199 (63.8%)
On target	11/13 (84.6%)	12/13 (92.3%)	13/13 (100%)	0/2 (0%)	9/12 (75%)	12/13 (92.3%)

[a] ARV drug supply continuity during the first year of the PASER-M survey. [b] Viral load as measured retrospectively for the PASER-M survey. [c] Patients who transferred out or died before the first pick-up were excluded from analysis. [d] No site dispensary. ART, antiretroviral therapy; ARV, antiretroviral; VL, viral load; NA, not applicable; WHO, world health organization.

for prevention of mother to child transmission, mono/dual therapy and/or ART) [19]. As shown in table 2, it was feasible to monitor 5 of the selected indicators at all sites. The EWI "on-time ARV drug pickups" was monitored at 2 sites. All patients at 11 of 13 (84.6%) sites were prescribed an initial first-line regimen according to WHO guidelines [21]. At site 9, 2 patients received first-line protease inhibitor (PI)–based regimens, and at site 12, an inappropriate triple-nucleoside reverse transcriptase inhibitor (NRTI) combination was prescribed.

Twelve (92.3%) sites met the targets of ≤20% patients LTFU, and all sites retained ≥70% patients on first-line ART after 12 months. The overall proportion of patients LTFU within the first year of ART was 9.8% across sites (range, 3.0%–21.4%). Inappropriate switch to PI-based or inappropriate substitution to triple-NRTI regimens within the first 12 months took place in 19 patients at 7 sites. A total of 19 patients at 8 sites were switched to second-line ART after suspected treatment failure.

Neither of the sites at which ARV drug pickups were monitored met the target for this indicator: 78% of patients at site 2 and 66% of patients at site 3 were on time for 2 consecutive pickups. When assessing pickups for the entire first year of ART, 4% of patients at site 2 and 6% of patients at site 3 were always on time (data not shown). Calculation of the MPR revealed that 86% of patients at site 2 and 59% of patients at site 3 were in possession of ARVs 100% of the time in the first year of ART. Antiretroviral drug supply was uninterrupted at 75% of sites. Three sites reported stockouts of fixed-dose combinations (FDCs), but not of individual ARV components, for a period of 1–3 months.

All but 1 site (92.3%) met the target of ≥70% of patients achieving VL suppression after 12 months of first-line ART. Overall, 76.0% of all patients in the cohort were classified as having VL suppression or HIVDR prevention. The remaining patients either had a VL >.1000 copies/mL (n = 166), had no available VL result (n = 148), were no longer on first-line ART (switch, n = 19; stop, n = 2), or were LTFU (n = 268) and were classified as having possible HIVDR.

Evaluation of adherence measures

As summarized in table 3, the indicator "on-time ARV drug pickup" had a sensitivity of 46.6% (95% CI, 35.9%–57.5%) and a specificity of 79.4% (95% CI, 74.6%–83.7%) for determining possible HIVDR after 12 months of first-line ART. An MPR of 100% yielded 67.0% (95% CI, 56.2%–76.7%) sensitivity and 83.5% (95% CI, 79.0%–87.4%) specificity. The positive predictive value of on-time ARV drug pickup and MPR were 38.3% (95% CI, 29.1%–48.2%) and 52.7% (95% CI, 43.0–62.2%), respectively. The positive likelihood ratios for the 2 measures were 1.6 and 3.9, respectively.

Table 3. Performance of on-time ARV drug pick-up and the medication possession ratio to determine possible HIV drug resistance[a] at 12 months

	Sensitivity (%)	Specificity (%)	PPV (%)	NPV (%)	PLR (%)	NLR (%)
On-time ARV drug pick-ups	46.6 (35.9-57.5) [41/88]	79.4 (74.6-83.7) [255/321]	38.3 (29.1-48.2) [41/107]	84.4 (79.8-88.3) [255/302]	1.6	0.8
100% MPR	67.0 (56.2-76.7) [59/88]	83.5 (79.0-87.4) [268/321]	52.7 (43.0-62.2) [59/112]	90.2 (86.3-93.4) [268/297]	3.9	0.4

Numbers in parentheses refer to the 95% confidence interval. Numbers in square brackets refer to the numerator and denominator from which the relevant percentages are calculated. Medication possession ratio is defined as the amount of time an individual is in possession of ARVs divided by the time between ARV prescriptions. [a] Possible HIVDR defined as patients with viral load > 1000 c/ml, patients lost to follow-up, patients who were no longer on appropriate first-line regimens (due to stopping or switching ART), or from whom no follow-up specimen was available. ARV, antiretroviral; MPR, medication possession ratio; PPV, positive predictive value; NPV, negative predictive value; PLR, positive likelihood ratio; NLR, negative likelihood ratio.

DISCUSSION

The assessment of the WHO-recommended EWIs as part of the ongoing PASER-M study at 13 public and nongovernmental ART sites in 6 African countries has identified vulnerable aspects of ART program functioning that are potentially associated with the emergence of HIVDR. Feedback to the respective sites is expected to trigger interventions targeted at the most at-risk populations. The measurement of the MPR, as a proxy measure for drug adherence, appeared to have better performance to predict possible HIVDR after 12 months of ART than the indicator "on-time ARV drug pickups."

Monitoring the indicator "on-time ARV drug pickups" was not feasible at all sites because patient or pharmacy records were found to be incomplete or could not be easily accessed even though the required data may have been routinely collected. The complexity associated with extracting the data required for the drug pickup indicator has been reported previously [5, 6]. The percentage of patients picking up ARVs on time for the entire first year of ART was unrealistically low, suggesting that this indicator may be too stringent or potentially signaling that pharmacy data may be unreliable. Notably, the indicator assessing 2 consecutive drug pickups after ART initiation achieved better performance in predicting virological outcomes. With a specificity of 79.4%, this cross-sectional indicator may be useful in correctly classifying persons in whom VL suppression will be achieved and could serve as a practical tool for clinics: adherence should be strongly reinforced in patients who fail to refill 2 drug pickups on time. Significantly, the MPR yielded higher sensitivity, specificity, and positive and negative predictive values than did ARV pickup information. As reflected by the positive likelihood ratio, patients with an MPR <100% were 3.9 times as likely to be classified as having possible HIVDR after 12 months of ART compared with patients with perfect medication possession. The MPR was therefore more accurate in identifying patients at risk of HIVDR development than pickup information alone, which had a positive likelihood ratio of 1.6.

The assessment of ART prescribing practices is relevant as inappropriate drug combinations have been documented to lead to the development of HIVDR [22]. The rate of correct prescribing was high, but did not reach 100% at all sites. One patient received an inappropriate triple-NRTI regimen for unknown reasons. Protease inhibitor–based first-line regimens were prescribed for 2 patients diagnosed with Kaposi sarcoma at site 9, in spite of the recommendation to reserve these drugs for second-line therapy in the setting of limited formularies [21].

Limiting attrition is essential because returning patients who were previously LTFU risk virological failure due to selected drug-resistant virus [23]. The attrition rate was lower

than has previously been reported in African ART programs [24] but varied substantially between sites. The site with the highest proportion of patients LTFU (21.4%) serves an urban, mobile population and performs limited patient tracing. Failure to correctly classify patients as deceased might have contributed to a high proportion presumed LTFU at both clinics that did not meet the indicator target. This underscores the importance of proper administration at ART sites for obtaining reliable EWI results. All sites met the target for patient retention on first-line ART at 12 months, which highlights the general effectiveness of first-line regimens and the success of managing toxicity with in-class substitutions. For 15 patients at 7 sites, inappropriate substitutions within first-line regimens to PI-based regimens occurred, limiting durable second-line options.

Because treatment interruptions may lead to HIVDR in resource-limited settings [25], it is essential that ARV drug supply continuity be monitored strictly. The sites that did not meet the indicator target had stockouts of FDCs that were replaced by the individual ARV components. Even though FDC stockouts lasted up to 3 months (site 9), they did not result in stopping or changing of individual patients' regimens.

Lastly, the optional indicator "VL suppression at 12 months" identified 1 clinic in which <70% of patients achieved HIVDR prevention. This poor performance was largely due to the high rate of patients LTFU at this clinic. Patient retention and tracing are the most important programmatic factors to be targeted for improvement at this site, which failed to meet 2 of 5 EWI targets.

The current study has some limitations. Because the PASER-M study used eligibility criteria, the population may not be completely representative of the general clinic population. However, additional selection bias is likely to be limited because all new patients who qualified for ART initiation were screened and enrolled in the study consecutively within a limited median time period of 12 months [19].

In conclusion, this pilot assessment of EWIs in the PASER clinical network has identified deficiencies in ART site functioning that should be targeted to minimize the emergence of preventable HIVDR. Nine sites failed to meet at least 1 EWI target; this should raise concern and flag those aspects of the clinic or pharmacy that need to be improved. This report provides the first example of how the WHO site-based EWIs, part of the WHO global strategy for HIVDR prevention and assessment, have been successfully abstracted and evaluated within an ongoing population-based monitoring study. Early warning indicators approximating drug adherence appeared to be helpful in identifying patients at risk for the development of HIVDR who would benefit from targeted adherence support. Based on our findings, the assessment of on-time refilling for 2 consecutive drug

pickups as opposed to the entire first year of ART was more feasible and informative. Our data suggest an additional advantage of the MPR over pickup information, because this measure was better able to predict possible HIVDR emergence. Further studies should focus on examining the MPR target or threshold necessary to prevent treatment failure and HIVDR. Based on its feasibility and performance, it is recommended to incorporate MPR in future EWI monitoring efforts to estimate patient adherence to ART. In addition to the ongoing laboratory-based studies to assess transmitted and acquired HIVDR within the PASER network, EWI monitoring will contribute to improved ART site functioning and quality of care for HIV-infected persons in sub-Saharan African countries.

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AFRIKA

Chapter 14

Cost-effectiveness of laboratory monitoring for management of HIV treatment in sub-Saharan Africa: a model-based analysis

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ABSTRACT

Objective

To compare the cost-effectiveness of three different strategies for long-term monitoring of antiretroviral therapy (ART) failure and regimen switching in sub-Saharan Africa: a symptom-based approach, or monitoring of either CD4 cell counts or plasma viral load (pVL).

Design

Markov model

Setting and participants

Hypothetical HIV-infected adult population who began first-line ART and subsequently had up to 6 years of follow-up.

Main outcome measures

Total cost, life expectancy and incremental cost-effectiveness ratio (ICER).

Results

A symptom-based approach yielded a life expectancy of 64.0 months at a total cost of US$ 4028 per person. All laboratory-based strategies, at testing intervals of 6 or 12 months, were cost-saving and improved life expectancy, compared with a symptom based approach. The life-expectancy gain was larger for pVL than for CD4 strategies at 6-monthly (2.3 and 0.9 months, respectively) and 12-monthly testing (2.0 and 0.8 months, respectively). Cost-savings of 6-monthly pVL or CD4 testing were similar (US$ 630 and 621, respectively), whereas 12-monthly CD4 cell counts were more cost-saving than 12-monthly pVL (US$ 1132 and 880, respectively). Testing every 12 months – rather than every 6 months – decreased the ICER by 102% for CD4 cell count and 67% for pVL. These findings were robust to a wide range of deterministic sensitivity analyses, but were sensitive to the specificity and costs of diagnostic tests.

Conclusion

Additional diagnostic costs are balanced by cost-savings from avoiding unnecessary switching due to misdiagnosis of ART failure. Routine pVL monitoring may be preferred as a replacement for CD4 cell counts because of its additional public health advantages in preventing drug-resistance, supporting adherence and reducing HIV transmission.

INTRODUCTION

Combination antiretroviral therapy (ART) has been shown to dramatically improve survival especially in sub-Saharan Africa [1], a region that continues to be the most affected by the global HIV/AIDS epidemic [2]. During the past decade, ART has been scaled-up as widely and rapidly as possible to reach millions of HIV-infected people in resource-limited countries, although access is not yet universal [3]. A proportion of patients receiving ART will inevitably experience therapy failure, mainly due to poor adherence, treatment interruptions or drug interactions, putting them at increased risk of HIV-related morbidity and mortality.

In most ART programmes in resource-limited settings, management of ART effectiveness and decisions to switch from first-line to second-line therapy are based on the occurrence of new HIV-related clinical disease and, if available, CD4 cell count changes, rather than on the basis of plasma viral load (pVL) which is the standard-of-care in high-income countries [4,5]. The DART trial in Uganda and Zimbabwe – which did not assess pVL testing – demonstrated that CD4 cell count monitoring in the first 5 years of ART yielded a small but significant benefit in terms of disease progression and mortality, probably owing to earlier switching to second-line ART, compared to a clinically driven approach [6]. Real-time pVL testing can more accurately identify people who are experiencing ART failure than CD4 cell counts or clinically driven monitoring, and several studies in sub-Saharan Africa have shown that WHO-defined clinical and immunological criteria poorly predict virological failure in both adults [7–10] and children [11] receiving first-line ART. pVL monitoring additionally avoids the incremental costs associated with unnecessary switches to more expensive second-line regimens in patients without real virological failure [12], prevents drug-resistance accumulation [12–14] and subsequent resistance transmission [15] and reduces HIV transmission [16]. Previous model based evaluations of cost-effectiveness have yielded conflicting results regarding the survival gains and cost effectiveness associated with diagnostic strategies based on CD4 cell counts alone, or additive approaches that combined CD4 and pVL assessments [17–19].

Routine laboratory monitoring of CD4 cell counts or pVL requires substantial investment in laboratory infrastructure, equipment, training and arrangements for maintenance and quality control. As this could divert resources away from scaling-up access to ART, it is critical to establish whether this would be cost-effective. Our aim was therefore to develop a model to assess the total cost, life expectancy and cost-effectiveness of three diagnostic strategies for long-term ART management in sub- Saharan Africa: a symptom-based approach, or monitoring of either CD4 cell counts or pVL, with diagnostic testing at 6 or 12 months intervals.

METHODS

Procedure

From the perspective of the WHO-recommended public health model of ART delivery in resource-limited countries, which includes standard first-line, second-line and third-line regimens and limited laboratory monitoring [20], we developed a Markov model using MS Excel 2010 to estimate life expectancy and costs for a hypothetical adult population with HIV infection who began first-line ART, in accordance with 2010 WHO guidelines. Figure 1 illustrates the states in the model and all possible transitions between them during each cycle. We used a cycle of 6 or 12 months duration to correspond with the typical interval between diagnostic evaluations in current clinical practice. The first-line regimen could be switched to a second-line regimen when the criterion for therapy failure was fulfilled, dependent on the diagnostic strategy. While being treated, a patient is considered either a 'success' or a 'failure' with respect to the current treatment, meaning that their pVL is either below or above a critical threshold. The diagnostic strategies differ in their

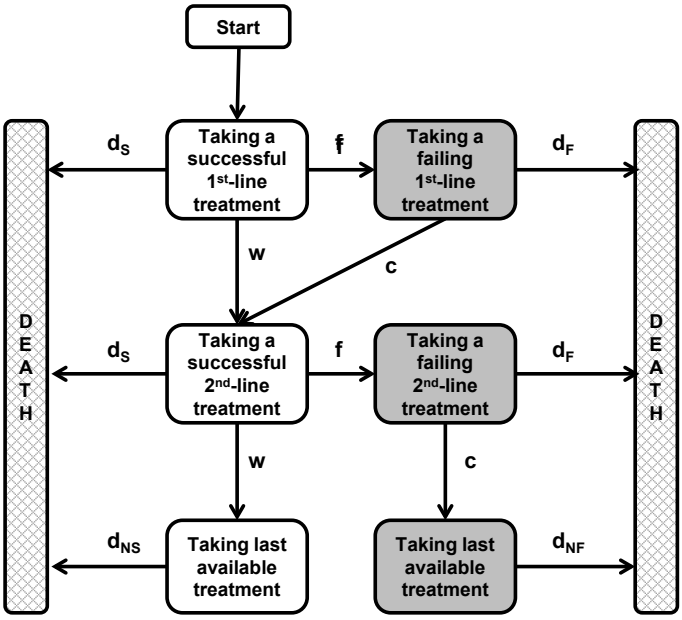

Figure 1: Markov model, a simplified transition-state diagram.
The probabilities in the model are: (f) the patient's viral load has risen above a critical threshold but this has not yet been detected and the patient is continuing on the failed therapy; (w) the patient has been wrongfully diagnosed as having failed therapy and has moved to the next line of therapy; (c) the patient's therapy failure is detected by either clinical judgment or a diagnostic test; (d_s) the patient dies while being successfully treated; (d_f) the patient dies while taking a failed therapy; (d_{NS}) the patient dies while not receiving further therapy after a false-positive diagnosis of therapy failure; and, (d_{NF}) the patient dies while not receiving further therapy after a true-positive diagnosis of therapy failure.

ability to detect treatment failures and to missclassify patients as treatment failures when they are actually responding to treatment. We ran our model to correspond to 6 years of follow-up and calculated the time spent in each health state. Attributing a cost to each health state, total costs and incremental cost-effectiveness ratios (ICERs) were estimated for each of the diagnostic strategies. We expressed our findings in terms of the total cost, life expectancy and the incremental ICER, that is incremental cost per life-year gained. In developing countries, an ICER of less than twice the per capita gross domestic product is generally thought cost-effective by policy makers (http://www.who.int/choice/en/).

For the symptom-based approach, therapy failure was defined as the occurrence of either a new or recurrent WHO-defined stage 3 or 4 clinical event at least 6 months after the start of ART [7]. For monitoring of CD4 cell count, therapy failure was defined as either a return to a CD4 cell count below the pre-therapy baseline, or a fall in CD4 cell count to less than 50% of the maximum value while on therapy [7]. Therapy failure by pVL criteria was defined as a value greater than 400 HIV RNA copies/ml, as this was the cut-off used in most published studies [21].

We assumed that the probability of treatment failure was highest during the first 3 months of ART and then declined over time. The initial probability of virological failure in the first 3 months of ART was based on a meta-analysis reported by Barth and et al. [21], computed as $1 - \sqrt{(1-22.0\%)} = 11.7\%$ (table 1). The subsequent probability of virological failure is believed to become constant with time [22]; as no data on limiting probability were available from Africa, we used the data from a UK cohort, computed as $1 - \sqrt[4]{(1-3.0\%)} = 0.76\%$ per three months [22]. Mortality in people receiving first-line ARTon data from the ART-LINC cohort was computed as $1 - \sqrt[4]{(1-2.2\%)} = 0.55\%$ per 3 months for those on a successful regimen and $1 - \sqrt[4]{(1-11.7\%)} = 3.1\%$ per 3 months for those who experienced first-line failure [23]. Mortality in people receiving second-line ART on data was estimated from the Médecins Sans Frontières ART programmes in Africa and Asia, as $1 - \sqrt[4]{(1-5.4\%)} = 1.4\%$ per 3 months [24]. We estimated mortality when no further treatment was available after a true or false diagnosis of failure as $1 - \sqrt[4]{(1-9.2\%)} = 2.4\%$ per 3 months [24] and used this same estimate for mortality if no treatment was available. We based the sensitivity and specificity of clinical judgment and CD4 cell counts for identifying virological failure on a study in South Africa by Mee et al. [7] (table 1).

Our analysis included the cost of diagnostic tests, ART and end-of-life care (table 1). The unit costs of first-line and second-line therapy were drawn from a costing study conducted at a large urban public sector site in Johannesburg, South Africa, in 2010 [25]. ART cost incorporated the cost of the most commonly used generic antiretroviral drugs

Table 1. Base-case model inputs

Variables	Data	Sources
Risk of treatment failure		
Initial probability of virological failure	11.7% per 3 months	Estimated with meta-analysis by Barth et al. (19)
Limiting probability of virological failure	0.76% per 3 months	Estimated with Benzie et al. (20)
Time to convergence	1.0 year	
Mortality		
Probability of death if successful regimen	0.55% per 3 months	Estimated with Keiser et al. (21)
Probability of death if first-line failure	3.1% per 3 months	As above
Probability of death if second-line failure	1.4% per 3 months	Estimated with Pujades-Rodriges et al. (22)
Probability of death if no treatment available, or no further treatment available after false diagnosis of failure	2.4% per 3 months	As above
Probability of death if no further treatment available after true diagnosis of failure	2.4% per 3 months	As above
Diagnosis inputs		
Clinical diagnosis		
False-positive proportion (1-specificity)	11.9%	Mee et al. (7)
False-negative proportion (1-sensitivity)	84.8%	As above
CD4 cell counts		
False-positive proportion (1-specificity)	4.2%	As above
False-negative proportion (1-sensitivity)	78.8%	As above
HIV viral load		
False-positive proportion (1-specificity)	0%	By definition
False-negative proportion (1-sensitivity)	0%	As above
Costs		
Unit cost of ART per patient per year		
First-line therapy	$ 403	Long et al. (23)
Second-line therapy	$ 1107	As above
Unit cost of laboratory test		
CD4 cell count	$ 9	Assumption based on South African public sector 2011
HIV plasma viral load	$ 36	Long et al. (23)
One-off cost of end-of-life care	$ 50	
Modelling period		
Cohort follow-up time	up to 6 years	

and other drugs, outpatient visits and infrastructure and other fixed costs and excluded inpatient care and laboratory tests. The all-in unit cost of a pVL test was drawn from the same study [25]. The all-in unit cost of a CD4 cell count was based on 2011 prices

in the public health sector in South Africa (W. Stevens, personal communication). All diagnostic costs accounted for equipment, consumables, maintenance and staff time. We assumed a one-off cost for end-of-life care, including hospitalization and illnesses. All costs are presented in US$ (November 2011).

Uncertainty

We performed deterministic sensitivity analyses to test the robustness of the model assumptions and data sources, using the point estimates of our model parameters. Sensitivity analyses considered a wide range of parameters in the model and varied therapy failure and mortality rates, sensitivity and specificity of diagnostic tests, costs of diagnostics, ART and end-of-life care and coverage of first-line, second-line and third-line ART. The sensitivity analyses included cost estimates of ART (2011) [26] and diagnostics (2009) [27] based on the latest Clinton HIV/AIDS Foundation (CHAI) prices. The lowest negotiated CHAI prices for the recommended first-line regimen of tenofovir, lamivudine, efavirenz (US$ 169 per year) and second-line regimen of atazanavir/ritonavir, tenofovir, lamivudine (US$ 395 per year) were used [26] and both estimates were increased by US$ 300 to cover for fixed costs of medical care. Both low-end and high-end CHAI estimates for diagnostic tests were considered [27].

RESULTS

Base-case analysis

In the base-case analysis, the symptom-based approach yielded a life expectancy of 64.0 months at a total cost of US$ 4028 per person. All laboratory-based diagnostic strategies, at testing intervals of either 6 or 12 months, were cost-saving and improved life expectancy, compared with a symptom-based approach (table 2 and figure 2). The gain in life expectancy was larger for pVL strategies than CD4 cell count strategies, at both 6 (2.3 and 0.9 months, respectively) and 12 months testing intervals (2.0 and 0.8 months, respectively). Cost-savings of pVL or CD4 cell counts every 6 months were similar (US$ -630 and -621, respectively), whereas CD4 testing every 12 months was more cost-saving than pVL testing every 12 months (US$ -1132 and -880, respectively). Compared with CD4 cell counts every 6 months, pVL testing every 12 months reduced costs by US$ 249. CD4 cell counts every 12 months was a more cost-effective strategy than CD4 cell counts every 6 months, or pVL testing every 6 or 12 months. For equivalent strategies, testing every 12 months rather than every 6 months was associated with a minimum life expectancy loss of 3 days for CD4 strategy and 11 days for pVL strategy, whereas the additional cost-savings were substantial (102% decrease in the ICER for CD4 cell count and 67% for pVL) (table 2).

Table 2. Base-case analysis results – cost-effectiveness

Monitoring strategy	Total life expectancy (months)	Total cost per person (US$)	Gain in life expectancy (months) *	Incremental costs per person (US$)*	ICER (US$/LYG)*
Symptom-based approach	64.0	4028	Reference	Reference	Reference
CD4-only every 6 months	64.9	3397	0.9	-630	-8024
CD4-only every 12 months	64.8	2896	0.8	-1132	-16193
pVL-only every 6 months	66.3	3407	2.3	-621	-3183
pVL-only every 12 months	66.0	3148	2.0	-880	-5319

pVL,plasma viral load; ICER, incremental cost effectiveness ratio, expressed as cost (US$) per life-years gained; na, not applicable.

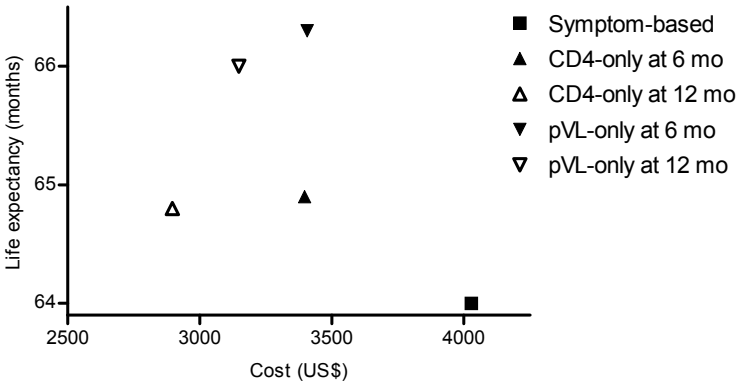

Figure 2. Base-case analysis results - cost-effectiveness.
Figure shows life-expectancy and total cost per patient over six years modeling period. All laboratory strategies were associated with higher life expectancy and reduced cost. A longer test interval substantively reduced cost. mo, months; pVL, viral load.

Sensitivity analysis

A wide range of sensitivity analyses varying the base-case assumptions and model parameters also showed that all laboratory-based diagnostic strategies were cost-saving, relative to a symptom-based approach (table 3). The superiority of laboratory-based diagnostic strategies was not sensitive to variations in mortality and failure rates.

The cost-effectiveness of laboratory-based diagnostic strategies was sensitive to the specificity, and to a lesser extent the sensitivity, of clinical judgment and the diagnostic tests. If the specificity of clinical judgement was increased to 95%, laboratory monitoring of either CD4 or pVL was cost-saving for 12-monthly intervals and cost-effective for 6-monthly intervals. Decreasing the specificity of CD4 to 90%, 6 or 12 monthly CD4 monitoring remained cost-saving. Decreasing the specificity of pVL testing to 95%, pVL

Table 3. Sensitivity analyses results

	Costs versus symptom-based approach				ICER versus symptom-based approach			
	CD4 6-monthly	CD4 12-monthly	pVL-only 6-monthly	pVL-only 12-monthly	CD4 6-monthly	CD4 12-monthly	pVL-only 6-monthly	pVL-only 12-monthly
Base-case (deterministic)	-630	-1132	-621	-880	-8024	-16193	-3183	-5319
Sensitivity analyses (deterministic)								
Failure rates								
SA1: initial probability increased by 50%	-515	-1045	-352	-602	-6463	-16389	-1585	-3179
SA2: initial probability decreased by 50%	-760	-1227	-930	-1186	-10011	-16413	-5940	-8839
SA3: limiting probability increased by 20%	-623	-1127	-599	-858	-8005	-16453	-3058	-5185
SA4: limiting probability decreased by 20%	-638	-1136	-644	-902	-8042	-15939	-3314	-5455
SA5: time to convergence set to 0.5 years	-699	-1182	-789	-1057	-8808	-14859	-4421	-6825
SA6: SA1 and SA3 combined	-509	-1041	-335	-586	-6442	-16634	-1508	-3093
SA7: SA2 and SA4 combined	-770	-1233	-961	-1215	-10020	-16133	-6190	-9076
Mortality rates								
SA8: probability of death if successful regimen increased by 20%	-625	-1123	-618	-875	-8641	-17702	-3395	-5688
SA9: probability of death if successful regimen decreased by 20%	-635	-1140	-624	-884	-7484	-14907	-2994	-4990
SA10: probability of death if first-line failure increased by 20%	-633	-1137	-609	-882	-7983	-17838	-2800	-4978
SA11: probability of death if first-line failure decreased by 20%	-628	-1126	-635	-879	-8104	-14714	-3721	-5782
SA12: probability of death if second-line failure increased by 20%	-627	-1126	-615	-874	-7700	-15093	-3071	-5132
SA13: probability of death if second-line failure decreased by 20%	-634	-1137	-627	-886	-8382	-17484	-3305	-5522
SA14: probability of death if no treatment available, or no further treatment available after true or false diagnosis of failure increased by 20%	-612	-1107	-603	-857	-6583	-12352	-2877	-4646

Table 3 (continued)

	Costs versus symptom-based approach				ICER versus symptom-based approach			
	CD4 6-monthly	**CD4 12-monthly**	**pVL-only 6-monthly**	**pVL-only 12-monthly**	**CD4 6-monthly**	**CD4 12-monthly**	**pVL-only 6-monthly**	**pVL-only 12-monthly**
SA15: probability of death if no treatment available, or no further treatment available after true or false diagnosis of failure decreased by 20%	-649	-1157	-640	-904	-10212	-23325	-3561	-6199
Sensitivity and specificity								
SA16: sensitivity and specificity CD4=VL=100%	-850	-995	-621	-880	-4358	-6013	-3183	-5319
SA17: sensitivity set to 100% for all three options	-667	-1034	-1008	-1266	-7580	-11927	-8241	-13680
SA18: clinical diagnosis specificity set to 80%	-1060	-1562	-1051	-1310	-7012	-10946	-3927	-5504
SA19: clinical diagnosis specificity set to 90%	-475	-976	-466	-724	-7707	-18400	-2614	-4880
SA20: clinical diagnosis specificity set to 95%	37	-464	47	-212	1500	-28267	331	-1894
SA21: clinical diagnosis sensitivity set to 10%	-534	-1035	-525	-784	-5699	-12156	-2496	-4339
SA22: clinical diagnosis sensitivity set to 20%	-704	-1205	-695	-954	-10617	-20874	-3803	-6230
SA23: CD4 specificity set to 90%	-17	-667	na	na	-513	-10382	na	na
SA24: CD4 specificity set to 100%	-1235	-1499	na	na	-12658	-21217	na	na
SA25: CD4 sensitivity set to 15%	-728	-1214	na	na	-12235	-20795	na	na
SA26: CD4 sensitivity set to 20%	-645	-1144	na	na	-8514	-16795	na	na
SA27: CD4 sensitivity set to 25%	-579	-1082	na	na	-6502	-14005	na	na
SA28: CD4 sensitivity set to 30%	-528	-1026	na	na	-5263	-11964	na	na
SA29: pVL specificity set to 95%	na	na	5405	-470	na	na	36	-2994
SA30: pVL sensitivity set to 95%	na	na	-628	-893	na	na	-3258	-5514
Diagnostic cost								
SA31: CD4 set to $ 2.50, VL set to $28 (CHAI low-end)	-679	-1159	-689	-914	-8633	-16558	-3531	-5525
SA32: CD4 set to $ 8, VL set to $29 (CHAI high-end)	-638	-1136	-680	-910	-8114	-16221	-3487	-5500

Table 3 (continued)

	Costs versus symptom-based approach				ICER versus symptom-based approach			
	CD4 6-monthly	CD4 12-monthly	pVL-only 6-monthly	pVL-only 12-monthly	CD4 6-monthly	CD4 12-monthly	pVL-only 6-monthly	pVL-only 12-monthly
SA33: pVL set to $100	na	na	-79	-606	na	na	-406	-3664
SA34: pVL set to $25	na	na	-714	-927	na	na	-3661	-5603
ART cost								
SA35: ART cost decreased by 20%	-491	-898	-437	-674	-6251	-12822	-2239	-4073
SA36: first-line set to $169, second-line set to $395 including drugs only (CHAI prices)	-155	-337	13	-175	-1971	-4815	68	-1057
SA37: first-line set to $469, second-line set to $695 including drugs and fixed costs of medical care ($300) (CHAI prices)	-128	-328	78	-131	-1629	-4689	401	-794
SA38: SA32 and SA37 combined including diagnostics, drugs and fixed costs (CHAI prices)	-135	-333	19	-161	-1724	-4750	97	-975
Cost of end-of-life care								
SA39: cost of end-of-life care doubled	-633	-1133	-625	-884	-8052	-16220	-3205	-5342
Line of ART with laboratory monitoring								
SA40: second-line monitoring added	-606	-1123	-547	-844	-7718	-16074	-2803	-5103
ART coverage								
SA41: second-line coverage set to 50%	-584	-1043	-565	-806	-4922	-6976	-2336	-3456
SA42: second-line coverage set to 10%	-546	-973	-521	-748	-3628	-4561	-1863	-2599
SA43: first-line set to 50%, second-line set to 10%	-273	-546	-260	-433	-3628	-5056	-1863	-2986
SA44: first-line set to 30%, second-line set to 1%	-161	-371	-153	-304	-3409	-5281	-1774	-3309
SA45: third-line coverage set to 100%, combined with SA40	-660	-1199	-602	-915	-18960	-155208	-3985	-8628

ICER, incremental cost-effectiveness ratio, expressed as cost (US$) per life-years gained; SA, sensitivity analysis; CHAI, Clinton HIV/AIDS Foundation; VL, HIV viral load.

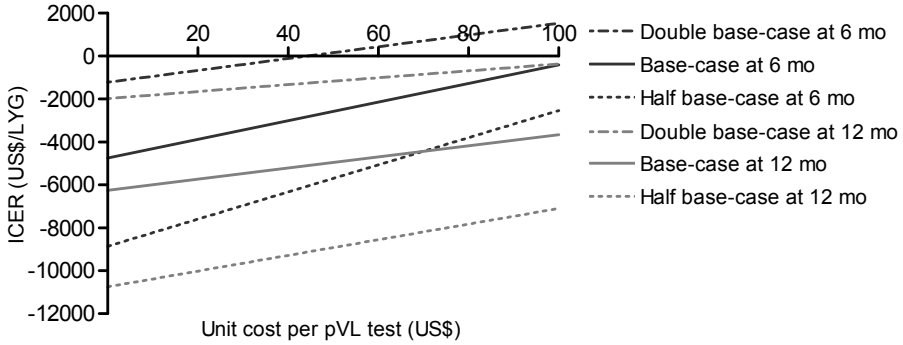

Figure 3. Sensitivity analysis of plasma viral load only monitoring strategies.
The ICER of pVL-only monitoring, compared with a symptom-based approach, is represented at the y-axis. Half or double base-case indicates half or double the rate of virologic failure, respectively, compared with the base-case. A negative ICER suggests that a strategy was cost-saving, compared with a symptom-based strategy. The ICER was sensitive to the per-test cost, the monitoring interval, and the rate of virologic failure. ICER, incremental cost-effectiveness ratio; LYG, life-year gained; pVL, plasma viral load.

monitoring was cost-saving for 12-monthly intervals and cost-effective for 6-monthly intervals.

The cost-effectiveness of laboratory-based diagnostic strategies was also somewhat sensitive to ART cost. In sensitivity analyses that used CHAI prices of ART, pVL testing every 6 months was cost-effective and all other laboratory-based strategies were cost-saving. Laboratory-based diagnostic strategies remained cost saving even if coverage of first and second line was restricted to as low as 30 and 1%, respectively. The cost-effectiveness of laboratory-based strategies was further increased if we assumed availability of third-line therapy, including diagnostic monitoring of the preceding second-line therapy.

The cost-effectiveness of pVL strategies was sensitive to diagnostic cost, monitoring interval and the rate of virological failure (figure 3). If the per test cost was increased to US$ 100, both the 6 and 12 monthly pVL strategies remained cost-saving. pVL strategies were more cost-saving than CD4 strategies if the pVL test cost was reduced. For instance, for 6-monthly test intervals, pVL was more cost-saving then CD4, at a pVL test cost of US$ 34 or less.

DISCUSSION

Our findings show that laboratory-based diagnostic monitoring, with either CD4 cell counts or pVL testing, is cost-saving for long-term ART management in sub-Saharan

Africa, compared with a symptom-based approach using WHO-defined clinical criteria. This conclusion is robust when assessed with a wide range of sensitivity analyses around the base-case. The savings with laboratory-based diagnostic strategies – despite additional diagnostic costs – were primarily because of the better performance (i.e. higher test specificity) at identifying and diagnosing those patients who have genuinely failing on their current therapy and who are eligible for regimen switch, so averting the incremental health expenditure of unnecessary switches to more expensive second-line regimens and intensified monitoring. For equivalent strategies, testing every 12 months rather than every 6 months substantially reduced cost, while not reducing life expectancy. This finding suggests that routine laboratory testing once every year may be sufficient, restricting more frequent testing to high-risk cases only.

Our findings seem robust for several reasons. First, varying diagnostic test performance, which may differ across assays, settings and populations [7–11], did not change our conclusions. Second, pVL strategies remained cost-saving even at substantially increased pVL test costs – which may be more realistic in other African countries. Third, the treatment costs we included were based on the most commonly used generic drug costs in South Africa and in the CHAI pricing list – this is a conservative assumption (i.e. it does not favour laboratory monitoring), as usual care possibly includes a wider range of more expensive drugs leading to underestimation of the benefits of more efficient diagnosis. Fourth, diagnostics can only be cost-effective when used in the context of treatment, whereas actual coverage of first-line, second-line and third-line ART differs between countries in the sub-Saharan African region [3]. Our base-case model demonstrated that laboratory-based diagnostic strategies were associated with cost-savings in settings with universal access to first-line and second-line ART. Additionally, sensitivity analyses showed that diagnostic monitoring was also cost-saving in settings where access to ART is still restricted. Therefore, our findings have value for health policymakers in countries that are close to achieving universal access (e.g. Namibia, Botswana and Rwanda), but also for countries with a less advanced ART scale-up [3]. Of note, the 2010 WHO ART guidelines recommend that national programs should develop policies for third-line therapy [20]; our model suggests that in settings where third-line coverage is assumed to be universal, diagnostic monitoring will reduce costs.

Although with current test cost estimates, 12-monthly CD4 monitoring may seem the most cost-effective strategy, there are a number of arguments that would favour pVL monitoring. First, the cost-effectiveness of pVL monitoring strongly depends on the test cost and the cost-effectiveness will further increase if the test price is reduced. Second, additional public health benefits of pVL monitoring not captured by the model might be valuable. These include fewer accumulation of drug-resistance mutations [13] and re-

duced subsequent resistance transmission [15], operational support tool for improving adherence [28], reduced incidence of opportunistic infections and mortality [29] with resultant increased economic productivity and reduced HIV transmission by limiting the number of persons with non-suppressed HIV replication [16]. Therefore, the model may underestimate the overall benefits of pVL testing. Third, challenges related to the implementation of pVL monitoring in resource-limited settings are increasingly becoming surmountable, as recent advances in pVL technology enable lower per-test cost as well as simpler machines that require less infrastructure, maintenance and technical expertise [30].

This model is the first cost-effectiveness analysis we know of that compares different diagnostic strategies for ART management that includes a pVL-only strategy – without concomitant CD4 cell counts. Previous studies in developed countries have suggested that there is limited benefit from continued measurements of CD4 cell counts in patients who have achieved full pVL suppression, which can be considered a marker for sustained high CD4 cell counts (>350 cells/μl) [31, 32]. Use of CD4 cell counts could thus be restricted to establish eligibility for ART initiation and to determine the need for prophylaxis for opportunistic infections in patients who have a detectable pVL during ART. Recently updated national ART guidelines in some countries (e.g. Malawi [33]) no longer recommend the use of CD4 cell counts for routine monitoring of patients on ART, instead they recommend routine pVL monitoring.

The feasibility and cost-effectiveness of pVL monitoring in the context of scaling-up ART in resource-limited settings have been debated [34–36], and WHO guidelines stipulate that pVL monitoring is desirable, but not essential, for a public health approach to ART [20]. Previous empirical research has suggested that routine pVL monitoring may yield limited survival benefit in the short-term [37,38]. Of note, a recent cluster randomized trial in Zambia found that routine 6-monthly pVL monitoring did not reduce all-cause mortality over the first 36 months of ART, compared with the current standard-of-care of using pVL sparingly to adjudicate discrepancies between CD4 and clinical assessments, although routine pVL monitoring resulted in earlier regimen change [39]. However, longer term follow-up data are not available. A previous modelling study, which used 2007 data from southern Africa, reported that immunological monitoring was cost-effective compared with symptom-driven strategies and that the addition of pVL monitoring led to a 2-month gain in life expectancy [17]. However, the ICER was less favourable for pVL than for CD4 cell count monitoring. Another modelling exercise also found that pVL monitoring provided a moderate long-term advantage, but the high associated costs were not deemed cost-effective for most resource-limited settings [18]. Our model, which was based on up-to-date cost data and included a pVL-only strategy – rather than

a more expensive additive diagnostic strategy – demonstrated a beneficial relative cost-effectiveness for laboratory-based diagnostic strategies.

The assumptions included in the model are a source of potential limitations. pVL testing was our reference standard for diagnosing therapy failure and was therefore apportioned 100% sensitivity and specificity. We acknowledge that very few diagnostic tests have perfect test performance. As might be expected, our conclusions based on the model were somewhat sensitive to variations in the sensitivity and specificity values. In addition, few published data were available to inform the estimates of costs related to the provision of healthcare, particularly outside of South Africa, which may limit the generalizability of the results to other settings. Publishing these costs in future studies would improve the accuracy of this and future economic analyses. Available data on second-line failure and mortality rates from resource-limited countries were somewhat limited, although the estimates we used concur with a recently published meta-analysis [40]. Finally, the current study did not assess paediatric populations; given that recent studies suggest that in children, CD4 cell counts perform even worse compared with pVL [11], it seems reasonable to assume that routine pVL monitoring will also be beneficial in children. Further research that would improve the model would include additional studies of the relative sensitivity and specificity of diagnostic testing in different subpopulations and settings, access to detailed data of healthcare costs in different countries and inclusion of the additional public-health advantages of pVL monitoring, particularly the potential impact of routine pVL monitoring in preventing secondary HIV infections [16].

In conclusion, we have identified that laboratory-based diagnostic strategies can provide substantial cost savings for long-term ART management in sub-Saharan Africa by averting the high costs of unnecessary switching to second-line therapy. pVL monitoring may be preferred over CD4 cell counts because of its important combined public-health advantages. Routine pVL monitoring, at least annually, in ART programmes should be seriously considered. As the number of persons receiving ART rises and test prices go down, the potential health benefit and cost-savings from the use of laboratory monitoring will further increase.

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Contributors

T.F.R.W., M.T. and A.M.H. conceived the study. A.W.S. designed and programmed the model, with input from R.L.H., T.F.R.W. and A.M.H. The cost-effectiveness analyses were undertaken by R.L.H. supported by A.W.S. and A.M.H. W.S. gave advice on the monitoring of HIV treatment and advised on issues particular to developing countries. R.L.H. wrote the first draft of the manuscript, with the assistance of A.W.S. and A.M.H. A.W.S., A.M.H., M.T. and T.F.R.W. helped with the interpretation of the results and critically reviewed and edited the paper. All authors read, commented on and approved the final manuscript.

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Chapter 15

Dried fluid spots for HIV type-1 viral load and resistance genotyping: a systematic review

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ABSTRACT

Background

Dried spots on filter paper made of whole blood (dried blood spots; DBS), plasma (dried plasma spots; DPS) or serum (dried serum spots) hold promise as an affordable and practical alternative specimen source to liquid plasma for HIV type-1 (HIV-1) viral load determination and drug resistance genotyping in the context of the rapidly expanding access to antiretroviral therapy (ART) for HIV-1-infected individuals in low- and middle-income countries. This report reviews the current evidence for their utility.

Methods

We systematically searched the English language literature published before 2009 on Medline, the websites of the World Health Organization and US Centers for Disease Control and Prevention, abstracts presented at relevant international conferences and references from relevant articles.

Results

Data indicate that HIV-1 viral load determination and resistance genotyping from DBS and DPS is feasible, yielding comparable test performances, even after storage. Limitations include reduced analytical sensitivity resulting from small analyte volumes (approximately 3.5 $\log_{10}$ copies/ ml at 50 µl sample volume), nucleic acid degradation under extreme environmental conditions, impaired efficiency of nucleic acid extraction, potential interference of archived proviral DNA in genotypes obtained from DBS and the excision of spots from the filters in high-volume testing.

Conclusions

This technology offers the advantages of a stable specimen matrix, ease of sample collection and shipment. The current sensitivity in drug resistance testing is appropriate for public health surveillance among pretreatment populations. However, consistently improved analytical sensitivity is needed for their routine application in the therapeutic monitoring of individuals receiving ART, particularly at the onset of treatment failure.

INTRODUCTION

The use of combination antiretroviral therapy (ART) in individuals infected with HIV type-1 (HIV-1) has been shown to effectively reduce morbidity and mortality worldwide [1]. The effectiveness of ART is typically assessed by regular enumeration of CD4+ T-cells and determination of HIV-1 viral load, and, in case of suspected treatment failure, drug resistance genotyping [2]. However, standard methods of viral load determination and genotyping require the appropriate collection, processing and storage of plasma specimens, trained personnel and a molecular laboratory infrastructure, including a centrifuge and freezers. High cost and complexity render these methods unsuitable for resource-limited settings [3]. Given that more than 90% of new HIV-1 infections occur in low- and middle-income countries, and that the availability of ART in these countries has greatly expanded in recent years [4], there is a need to develop simplified methods of specimen collection, storage and transport, which are adapted to field conditions.

The most promising approach in this respect is the spotting and drying of blood specimens on an absorbent filter paper matrix. This has several technical, practical and economic advantages over using liquid plasma. Dried blood spots (DBS) can be prepared by healthcare practitioners with relatively little training, require no manipulation at the clinic level, are non-hazardous and can be dispatched to reference testing facilities by regular mail at ambient temperature without the need for expensive dry ice shipments. Moreover, DBS are particularly attractive for paediatric applications, given the challenges of phlebotomy in young children. DBS are already being used for a number of serological and molecular (qualitative) assays, such as for screening of metabolic disorders in neonates [5, 6], detection of HIV-1 antibodies [7–9] and DNA PCR for infant diagnosis of HIV-1 [10–12]. Additionally, DBS as well as dried serum spots (DSS) and/or dried plasma spots (DPS) have been evaluated for HIV-1 viral load quantification [13–26], resistance genotyping [27–38], p24 antigen quantitation [39, 40] and CD4+ T-cell enumeration [41].

Compared with liquid plasma-based methods, however, the use of dried fluid spots has some potential disadvantages, which include reduced test sensitivity in HIV-1 viral load quantification [15, 23] and genotyping assays [30, 32, 33, 35] because of small analyte input volumes and impaired efficiency of nucleic acid extraction [29], as well as nucleic acid degradation under environmental storage conditions [16, 27, 32]. Moreover, archived proviral DNA *pol* sequences might interfere with the genotypic profiles generated from DBS [27, 33, 34].

This report reviews the current evidence for the utility of dried fluid spots as a specimen matrix for HIV-1 viral load and resistance genotyping assays. Additionally, remaining challenges and recommendations for further research are discussed.

METHODS

Search strategy and selection criteria

References for this review were identified by a systematic search of the English language literature published before 1 January 2009 on Medline, the websites of the World Health Organization (WHO) and US Centers for Disease Control and Prevention, abstracts presented at relevant international conferences and references from relevant articles. Search terms used in combination were 'HIV-1', 'diagnostic test', 'dried fluid spot', 'dried blood spot', 'dried plasma spot', 'dried serum spot', 'filter paper', 'viral load', 'resistance genotyping' and 'resource-limited settings'. Two observers independently reviewed and extracted data from the studies. Disagreements about data extraction were settled by conversation. Studies that evaluated the test performance of DBS, DPS and/or DSS for HIV-1 viral load quantification and/or genotyping were selected. Studies that evaluated the performance of dried fluid spots for diagnosis of HIV-1 infection using qualitative molecular methods were excluded. Extracted data were the laboratory methods used, test performance of dried fluid spots compared with the reference standard liquid plasma, and nucleic acid stability during storage. All RNA values are expressed as $\log_{10}$ transformed copy numbers of RNA per ml of liquid plasma, DBS, DPS or DSS equivalent.

HIV-1 VIRAL LOAD QUANTIFICATION

Dried blood spots

Laboratory methods

Twelve studies evaluated DBS for HIV-1 viral load quantification [13–21, 24–26] (table 1). All studies used 903 filter paper (Whatman, Maidstone, UK; previously Schleicher & Schuell, Keene, NH, USA), two of which additionally used Isocode paper (catalogue number 495020; Schleicher & Schuell) [16, 20]. DBS were prepared using whole blood, either obtained by venipuncture (anti-coagulated) [13, 14, 16, 17, 19, 20, 26], by finger puncture [18] or unspecified [15, 21, 24, 25]. The specimen input volumes were 50 µl [19, 20, 26], 100 µl [13, 16, 17, 25], 200 µl [14] or unspecified [18, 21, 24]. Viral load assays used were based on nucleic acid sequence based amplification (NASBA) or reverse transcriptase (RT)-PCR (table 1).

Table 1. Summary of studies that evaluated the use of DBS for HIV viral load quantification.

Reference	Year of publication	Laboratory methods								Test performance			Nucleic acid stability				Comment
		Specimen source	No.	Viral load range#	Filter paper	Spot volume (µl)	Drying time	Quantification	Elution-extraction	Correlation coefficient*	Mean RNA difference#	LDL#	Storage conditions	Correlation coefficient*	Mean RNA difference#	LDL#	
Abdo et al. (26)	2008	ARV experienced, South Africa	200	1.7 to 4.0	903	50	N/A	NucliSens EasyQ NASBA	EasyMAG	N/A	0.35	3.6	6wks at -20°C, 4°C, 28°C, 40°C	N/A	N/A	N/A	
Abravaya et al. (25)	2008	N/A	N/A	3 to 6	903	100	N/A	Abbott RealTime RT-PCR	Abbott RealTime RT-PCR	N/A	N/A	3.3	N/A	N/A	N/A	N/A	
Alvarez Munoz et al. (13)	2005	ARV naive, Mexico	127	N/A	903	100	overnight	NucliSens QT NASBA	Nuclisens	0.99	N/A	N/A	travel (3-27h) at room temperature; 7 days at 4, 22, and 37°C	0.95@	<0.5	N/A	Good correlation DBS-DPS after 7 days storage at 37°C
Ayele et al. (14)	2007	ARV naïve and experienced, The Netherlands and Ethiopia	103	<1.7 to 6.5	903	200	30 min	Rainbow NASBA	Boom et al. (50)	0.7706	N/A	N/A	N/A	N/A	N/A	N/A	Good correlation DBS-DPS
Brambilla et al. (15)	2003	US	6	3.6 to 5.7	903	50	overnight	NucliSens QT NASBA/ Amplicor RT-PCR (modified)	NucliSens/ CORD reagent	N/A	-0.44 to 0.82	3.6	52 weeks at room temperature and -70°C	N/A	-2.4%	N/A	Amplicor has a broader dynamic range and is more sensitive compared with NucliSens
Fiscus et al. (16)	1998	US	76	N/A	903†	100	overnight	NucliSens QT NASBA	NucliSens	0.88‡	0.0478‡	N/A	28 days at room temperature	N/A	-0.026 per day	N/A	Problems with stability may exist with prolonged storage

Table 1 (continued)

Reference	Year of publication	Laboratory methods							Test performance			Nucleic acid stability				Comment
		Specimen source	No.	Viral load range[#]	Filter paper	Spot volume (µl)	Drying time	Quantification	Elution-extraction	Correlation coefficient*	Mean RNA difference[#]	LDL[#]	Storage conditions	Correlation coefficient*	Mean RNA difference[#]	LDL[#]	
Idigbe et al. (24)	2003	Nigeria	94	N/A	903	N/A	N/A	Rainbow NASBA	Boom et al. (50)	0.999	N/A	N/A	N/A	N/A	N/A	N/A	
Mwaba et al. (18)	2003	ARV naïve, Zambia	51	N/A	903	N/A	3h	NucliSens QT NASBA	NucliSens	N/A	N/A	N/A	6 weeks at room temperature	N/A	N/A	3.5	Good correlation DBS-DPS
O,Shea et al. (19)	1999	UK	27	N/A	903	50	2h	NucliSens QT NASBA	NucliSens	0.84	<0.5	N/A	1 to 13 months at -70 °C	N/A	<0.5	N/A	
Kane et al. (17)	2008	Senegal	41	2.9 to 5.4	903	100	overnight	NucliSens EasyQ real-time	NucliSens-Boom et al. (50)	0.90	N/A	2.9	8 and 15 days at 37°C	0.98, 0.94, resp.	-0.07, -0.01, resp.	N/A	
Uttayamakul et al. (20)	2005	Thailand	30	N/A	903[†]	50	18-24h	NucliSens QT NASBA	NucliSens-Boom et al. (50)	0.817	0.174	N/A	N/A	N/A	N/A	N/A	
Waters et al. (21)	2007	ARV experienced, Uganda	306	2.7 to 5.7	903	N/A	N/A	COBAS TaqMan	N/A	0.72	N/A	N/A	N/A	N/A	N/A	N/A	High rate of false-positive viral loads

For each study a brief description is provided of laboratory methods used, DBS test performance compared with the reference standard liquid plasma (unless stated otherwise), data on nucleic acid stability under environmental storage conditions, and any relevant comments. * Correlation coefficients express correlation with the reference standard liquid plasma; [#] HIV viral load expressed as $\log_{10}$ copies/ml; [†] In addition to 903 filter paper, Isocode was used; [‡] Results are for 903 filter paper; for Isocode correlation coefficient = 0.90 and mean RNA difference = 0.22 $\log_{10}$ copies/ml; [@] For 7 days at 4, 22, and 37°C Kappa = 0.98, 0.83 and 0.94, resp. ARV, antiretroviral; LDL, lower detection limit; NA, not available; NASBA, nucleic acid sequence based amplification; RT, reverse transcriptase.

Test performance

All commercial viral load assays show the ability to quantify RNA from DBS, with a lower detection limit of 2.9 [17] to 3.3 [25] $\log_{10}$ RNA copies/ml at a specimen input volume of 100 µl, and 3.6 $\log_{10}$ copies/ml at a specimen input volume of 50 µl [15, 26]. Collectively, correlation coefficients for RNA viral loads in DBS and paired liquid plasma specimens ranged from 0.72 to 0.99 [13, 14, 16, 17, 19–21, 24]. In various studies, detection of RNA from DBS was linear over a broad dynamic range: 2.7 to 5.7 [21], 2.9 to 5.4 [17], 3.0 to 6.0 [25] and 3.6 to 5.7 [15] $\log_{10}$ copies/ ml. Median RNA differences between plasma and DBS were <0.5 $\log_{10}$ copies/ml [15, 16, 19, 20, 26] or unspecified [13, 14, 17, 18, 21, 24, 25]. One study reported a high rate of false-positive detectable RNA loads from DBS compared with plasma, which the authors attributed to a possible cross-reaction between cell-associated HIV-1 DNA and the RT-PCR assay [21]. One study reported a broader dynamic range and increased sensitivity at lower RNA loads for the Amplicor RT-PCR assay compared with the NucliSens NASBA assay, probably resulting from the differences in the amount of the actual RNA eluate that was used in the amplification steps for the two assays. For Amplicor, the specimen input volume was equivalent to 25 µl, which is comparable to the volume used for the standard Amplicor assay, whereas the specimen input volume for NucliSens was equivalent to 5 µl, which is 1/4 to 1/20 of the volume used for plasma analysis by the NucliSens assay [15] (table 1).

Nucleic acid stability

Several studies have shown that RNA in DBS is sufficiently stable under variable conditions: 7 days at 37°C and 60% humidity, 12 weeks at 22°C or freeze-thawing twice [23]; 15 days at 37°C [17]; 2 weeks at 22°C or 7 days at 37°C [13]; 3–27 h travel time at room temperature in different climates [13]; 6 weeks at room temperature (22–28°C) [18,26]; and 52 weeks at room temperature [15] or at -70°C [15,19]. However, Fiscus *et al*. [16] reported a statistically significant decrease of 0.0261 $\log_{10}$ copies/ml per day over a 28-day period at room temperature, which is equivalent to a loss of approximately 5% per day; in this study it was not clear whether the RNA degraded with time or whether there was increasing difficulty in recovering the RNA from the filter paper after prolonged storage (table 1).

Dried plasma spots

Laboratory methods

Nine studies evaluated DPS [13–15, 18, 21–24, 26] (and no study evaluated DSS) for RNA viral load quantification (table 2). All studies used 903 filter paper. The specimen input volumes were 50 µl [15, 23, 26], 100 µl [13], 200 µl [14, 22] or not specified [18, 21, 24]. Viral load assays used were based on NASBA or RT-PCR (table 2).

Test performance

All commercial viral load assays show the ability to quantify RNA from DPS, with a lower detection limit of 3.5 [23] to 3.6 [26] $\log_{10}$ copies/ml at a specimen input volume of 50 µl, and 3 $\log_{10}$ copies/ml at a specimen input volume of 200 µl [22]. Collectively, correlation coefficients for RNA levels in DPS and paired liquid plasma specimens ranged from 0.86 to 0.97 [14, 22–24]. In various studies, detection of RNA from DPS was linear over a broad dynamic range: 3.2 to 8.4 [23], 2.6 to 5.7 [22] and 3.6 to 5.7 [15] $\log_{10}$ copies/ml. Median RNA differences between plasma and DPS were 0.077 $\log_{10}$ copies/ ml [23], 0.16 $\log_{10}$ copies/ml [26], 0.64 $\log_{10}$ copies/ml [22] or unspecified [13–15, 18, 21, 24] (table 2).

Nucleic acid stability

Several studies have shown that for viral load determination RNA in DPS is sufficiently stable under variable conditions: 1 week at 4°C, 22°C [22] or 37°C [13]; 2 weeks at 4°C or 20°C [23]; 3 days at 37°C with high humidity [23]; 6 weeks at room temperature (22–28°C) [18, 26]; 1 year at room temperature or -70°C [15]. However, Amellal *et al*. [22] reported a significant loss of RNA (0.92 $\log_{10}$ copies/ml) in DPS stored at 37°C for 1 week compared with plasma at -80°C. As the greatest RNA depletion occurred in the experiment with the longest drying time, the authors speculated that the loss might be attributable to the absence of desiccant during storage (table 2).

Correlation of dried blood spots and dried plasma spots

Five studies evaluated the correlation of DBS and DPS for RNA quantification [13–15, 18, 21]. Significant correlations were found by most studies [13, 14, 18, 21], including evaluations done after storage for 6 weeks at 22–28°C [18] and for 7 days at 37°C [13]. Waters *et al*. [21] found that agreement between 122 DBS/ DPS pairs was fair (Cohen's Kappa=73%). One study reported that RNA loads from DBS were, on average, 0.11 $\log_{10}$ copies/ml (29%) higher than those from DPS [15], whereas Ayele *et al*. [14] found that RNA loads generated from DBS tended to be slightly lower than those from DPS.

RESISTANCE GENOTYPING

Dried blood spots

Laboratory methods

Nine studies evaluated DBS for HIV-1 genotyping [27, 28, 31–34, 36–38], one of which sequenced the cellular proviral DNA instead of viral RNA [36] (table 3). All studies used 903 filter paper, one of which additionally used the FTA matrix (Whatman) [28]. DBS were prepared using whole blood, either obtained by venipuncture (anti-coagulated)

Table 2. Summary of studies that evaluated the use of DPS/DSS for HIV viral load quantification

Reference	Year of publication	Laboratory methods							Test performance			Nucleic acid stability				Comment
		Specimens	No.	Viral load range#	Filter paper	Spot volume (µl)	Drying time	Quantification	Elution-extraction	Correlation coefficient*	Mean RNA difference#	LDL#	Storage conditions	Correlation coefficient*	Mean RNA difference#	LDL#	
Abdo et al. (26)	2008	ARV experienced, South Africa	200	1.7 to 4.0	903	50	N/A	NucliSens EasyQ NASBA	EasyMAG	N/A	0.16	3.6	N/A	N/A	N/A	N/A	
Alvarez Munoz et al. (13)	2005	ARV naive, Mexico	127	N/A	903	100	overnight	NucliSens QT NASBA	NucliSens	N/A	N/A	N/A	travel (3-27h) at room temperature; 7 days at 37°C	N/A	-0.19	N/A	Good correlation DBS-DPS after 7 days storage at 37°C
Amellal et al. (22)	2007	ARV naïve and experienced, France	45	2.6 to 5.7	903	200	4h	Amplicor RT-PCR	PBS buffer	0.92	-0.64	3	7 days at 4, 22 and 37°C, resp.	0.98, 0.97, 0.84, resp.	-0.20, 0.03, -0.92, resp.	N/A	Significant RNA loss after 7 days at 37°C, but not at room temperature. RNA loss associated with lack of desiccation.
Ayele et al. (14)	2007	ARV naïve and experienced, The Netherlands and Ethiopia	103	<1.7 to 6.5	903	200	30 min	Rainbow NASBA	Boom et al. (50)	0.92 /0.96†	N/A	N/A	N/A	N/A	N/A	N/A	Good correlation DBS-DPS
Brambilla et al. (15)	2003	US	6	3.6 to 5.7	903	50	overnight	NucliSens QT NASBA/ Amplicor RT-PCR (modified)	NucliSens/ CORD reagent	N/A	N/A	3.6	52 weeks at room temperature and -70°C	N/A	-2.4%	N/A	Amplicor has a broader dynamic range and is more sensitive compared with NucliSens

Table 2 (continued)

Reference	Year of publication	Laboratory methods								Test performance			Nucleic acid stability				Comment
		Specimens	No.	Viral load range#	Filter paper	Spot volume (µl)	Drying time	Quantification	Elution-extraction	Correlation coefficient*	Mean RNA difference#	LDL#	Storage conditions	Correlation coefficient*	Mean RNA difference#	LDL#	
Cassol et al. (23)	1997	ARV naïve, Canada	73	3.2 to 8.4	903	50	3h	NucliSens QT NASBA/ Amplicor RT-PCR (modified)	NucliSens/ Amplicor	0.97	-0.077	3	16 days at 4 and 22C, low-humidity; 3 days at 37°C high-humidity, resp.	0.98, 0.97, 0.97, resp.	-0.063, -0.080, -0.055, resp	N/A	Good correlation NucliSens-Amplicor over a 5 $\log_{10}$ dynamic range
Idigbe et al. (24)	2003	Nigeria	94	N/A	903	N/A	N/A	Rainbow NASBA	Boom et al. (50)	0.858	N/A	N/A	N/A	N/A	N/A	N/A	
Mwaba et al. (18)	2003	ARV naïve, Zambia	51	N/A	903	N/A	3h	NucliSens QT NASBA	N/A	N/A	N/A	N/A	6 weeks at room temperature	N/A	N/A	3.2	Good correlation DBS-DPS
Waters et al. (21)	2007	ARV experienced, Uganda	218	2.7 to 5.7	903	N/A	N/A	COBAS TaqMan	N/A	N/A	N/A	N/A	N/A	N/A	N/A	N/A	Excellent agreement with reference standard

For each study a brief description is provided of laboratory methods used, DPS/DSS test performance compared with the reference standard liquid plasma (unless stated otherwise), data on nucleic acid stability under environmental storage conditions, and any relevant comments. * Correlation coefficients express correlation with the reference standard liquid plasma; # HIV viral load expressed as $\log_{10}$ copies/ml; † Refers to non-B/B subtypes, respectively. ARV, antiretroviral; LDL, lower detection limit; NA, not available; NASBA, nucleic acid sequence-based amplification; PBS, phosphate-buffered saline; RT, reverse transcriptase.

Table 3. Summary of studies that evaluated the use of DBS for resistance genotyping

Reference	Year of publication	Laboratory methods								Test performance			Nucleic acid stability	Comment
		Specimen source	No.	Viral load range[#]	Subtypes	Filter paper	Spot volume (µl)	Drying time	Elution-extraction	Genotyping	Amplification success	Nucleotides*	Mutations*	Storage conditions	
Bertagnolio et al. (27)	2007	ARV naïve, Mexico	103	3.7 to 5	B	903	100	3h	NucliSens	In-house nested RT-PCR; PR-RT >1008 bp (2 amplicons),	78% overall; 94% >4log10 cps/ml	99.9%	92% (12/13)	3 months at 37°C and 85% humidity	
Buckton et al. (28)	2008	UK	33	1.9 to 5.1	multiple	903[†]	100	1h	Boom et al.(50)	PR-RT 1245 bp (2 amplicons)	83%boverall; 62% <1.7 log10cps/ml	N/A	83% (10/12)	3 months at 4°C and room temperature, with desiccant	Best DNA recovery with 903 and silica/ guanidine extraction
Garrido et al. (31)	2008	ARV experienced, Angola	77	<3 to 5.9	multiple	903	N/A	overnight	Boom et al. (50)	In-house nested RT-PCR; RT-gp41	58% overall	N/A	N/A	4°C, no desiccant	Poor amplification rate because of high HIV genetic diversity and lack of humidity control
Hallack et al. (32)	2008	ARV naïve and experienced, USA	33	3.1 to 5.6	B	903	75-80	4-24h	NucliSens	Trugene (1.3kb)	79% overall; 91% >3.8 log10 cps/ml; 58% 3-3.8log10 cps/ml	99.3%	100%	N/A	Sensitivity improved using 2 spots instead of 1
Masciotra et al. (33)	2007	ARV naïve and experienced, Spain	60	1.9 to 5.8	B	903	50	overnight	NucliSens/ Boom et al. (50)	ViroSeq/ Trugene (DNA: in-house nested RT-PCR; PR-RT 1023 bp)	83% overall; 100%>3.3 log10 cps/ml; 55% <3.3 log10 cps/ml	98.%	97% (306/316)	18-26 weeks at -20°C, with desiccant	HIV DNA in 44% of DBS
McNulty et al. (34)	2007	ARV naive, USA	9	3.5 to 5.5	B	903	50	overnight	Nuclisens/ Boom et al. (50)	In-house nested RT-PCR; PR-RT 1023 bp	N/A	98.6%	82% (14/17)	5-6 yrs at -30°C, -70°C, room temperature	

Table 3 (continued)

Reference	Year of publication	Laboratory methods								Test performance			Nucleic acid stability	Comment
		Specimen source	No.	Viral load range#	Sub-types	Filter paper	Spot volume (µl)	Drying time	Elution-extraction	Genotyping	Amplification success	Nucleo-tides*	Muta-tions*	Storage condi-tions	
Idem		ARV naive, Cameroon	40	2.8 to 5.3	multiple	idem	idem	idem	idem	idem	93% overall; 73% <4 log10 cps/ml; 100% >4 log10 cps/ml	98.5%	N/A	2-3 year at -20°C	
Steegen et al. (36)	2007	ARV naïve and experienced, Kenya	60	N/A	A, D, AD, G	903	50	overnight	QIAamp DNA blood mini kit (Qiagen)	In-house nested RT-PCR; PR 458 bp, RT 646 bp	93% overall; 55% <1.7 log10 cps/ml	97.9%	44% (15/34)	N/A	RNA sequencing superior to DNA sequencing for detection of resistance mutations
Youngpai-roj et al. (37)	2008	ARV-experienced, Spain	40	2.7 to 5.8	B	903	50	overnight	NucliSens	ViroSeq (1.8kb)	58% overall; 96% >4 log10 cps/ml	N/A	97% (158/163)	1year at 4°C, with desiccant	ViroSeq shows poor amplification
idem		idem	idem	idem	idem	idem	idem	idem	idem	In-house nested RT-PCR; PR-RT 1023 bp	95% overall	N/A	95% (275/291)	idem	
Ziemniak et al. (38)	2006	ARN naïve and experienced, US and Zambia	12	2.3 to 6.5	B, C	903	250	overnight	High Pure Viral Nucleic Acid Kit (Roche)	In-house nested RT-PCR; RT 663 bp	100% overall	N/A	100% (6/6)	<4.9 months at room temperature, with desiccant	

For each study a brief description is provided of laboratory methods used, DBS test performance compared with the reference standard liquid plasma (unless stated otherwise), data on nucleic acid stability under environmental storage conditions, and any relevant comments. * Concordance with the reference standard liquid plasma; # HIV viral load expressed as $\log_{10}$ copies/ml; † In addition to 903 filter paper, the FTA matrix was used. ARV, antiretroviral; N/A, not available.

[27, 32, 34, 36], by finger puncture [31] or unspecified [33, 37, 38]. The specimen input volumes were 50 µl [34, 36, 37], 75–80 µl [32], 100 µl [27], 250 µl [38] or not specified [31]. The studies include a number of different nucleic acid extraction and amplification protocols, including commercial kit-based strategies [33, 37] and in-house techniques [27, 31, 32, 34, 36, 38] (table 3).

Test performance

Overall, amplification success rates ranged from 58% to 95% [27, 31–34, 36–38]. Amplification success rates tend to be high for high RNA viral loads (>4 [27, 34, 37], >3.8 [32], >3.3 [33] or >3 [28] $\log_{10}$ copies/ml), but reduced for lower viral loads [28, 32, 33]. Buckton *et al*. [28] achieved a 100% amplification success rate from DBS with >3.0 $\log_{10}$ copies/ml and 62% from DBS with undetectable (<1.7 $\log_{10}$ copies/ml) plasma viral load at a 100 µl specimen input volume, using 903 filter paper and silica/guanidine extraction (table 3). Most studies have aimed to compare the nucleotide sequences generated from paired DBS and plasma specimens. Reported concordance between nucleotide sequences generated from the two specimen types ranged from 98.5% to 99.9% [27, 34]. Although some studies also reported concordance for drug resistance-associated codons between paired DBS and plasma specimens [27, 32, 34, 38], others have reported discrepancies [28, 33, 36]. A small number of studies examined the potential interferences of archived proviral DNA in the genotypic results generated from DBS. These studies were performed by amplifying from viral extracts with and without a reverse transcription step. The rate of amplification from DBS without reverse transcription ranged between 44% and 80% [27, 33, 34], which suggests that proviral DNA might contribute to a significant proportion of DBS consensus sequences.

Nucleic acid stability

Several studies have shown high success of amplification and genotyping after DBS storage under variable conditions: 3 months at 37°C and 85% humidity [27]; 18–26 weeks at -20°C with desiccant [33]; 2–3 years at -20°C or 6 years at -30°C [34]; 1 year at 4°C with desiccant [37]; and up to 4.9 months at room temperature with desiccant [38]. Garrido *et al*. [31] found impaired success of amplification resulting from lack of humidity control. Most studies highlighted the importance of drying DBS completely prior to storage in a zip-locked plastic bag containing silica gel desiccant. Following 1 year of storage at 4°C, Youngpairoj *et al*. [37] found an excellent (95%) amplification rate using an in-house assay, but poor (58%) amplification using ViroSeq, possibly because of the fact that ViroSeq amplifies a large *pol* fragment, which might be more sensitive to nucleic acid degradation during long-term storage at suboptimal temperature, humidity or both. McNulty *et al*. [34] found that none of the DBS stored for 5 years at room temperature were amplified. Youngpairoj *et al*. [37] showed that it is possible to overcome potential

losses in RNA integrity and efficiently genotype from DBS stored at 4°C by using nested RT-PCR that amplifies a smaller fragment (table 3).

Dried plasma/serum spots

Laboratory methods

Two studies evaluated DSS [29, 35] and two studies evaluated DPS [29, 30] for HIV-1 genotyping (table 4). All studies used 903 filter paper. The specimen input volumes was 20 µl [29, 30, 35]. Amplification was done by an in-house nested RT-PCR assay. Comparison of two elution–extraction methods showed the importance of the choice of extraction buffer; in particular, extraction lysis buffer applied directly to the spots led to aggregation of the filter paper, which probably impaired the elution efficiency [29] (table 4).

Test performance

Genotyping was reliable above viral load values of 4 $\log_{10}$ copies/ml from 20 µl DSS [35] and DPS [30]. The authors attributed the reduced sensitivity to the small sample volume used for the spot (20 µl) instead of 140 µl for a standard plasma sample (table 4).

Nucleic acid stability

High success of amplification and genotyping was achieved after storage of no more than 14 days at 4°C without desiccant [35] and 7 days at room temperature [29, 30]. More extreme conditions were not examined (table 4).

DISCUSSION

Most studies published to date have indicated that HIV-1 viral load quantification [13–26] and resistance genotyping [27–38] from dried fluid spots is feasible. Overall, DBS and DPS seem to yield comparable performance, even after storage [13–15, 18, 21]. However, certain limitations and challenges to their practical use remain. In the first place, the lower limit of detection of a viral load assay with dried fluid spots might never reach that of an assay with liquid plasma as a result of the small sample volumes of the spots. The currently reached sensitivity (approximately 3.5 $\log_{10}$ copies/ ml at 50 µl input volume) might still be useful to provide clinical guidance regarding drug regimen switch in individuals receiving ART. The current sensitivity levels in drug resistance testing seem to be appropriate for public health surveillance among newly diagnosed or pretreatment populations. This has led WHO to recommend the use of DBS for this application [42]. However, consistently improved analytical sensitivity is needed for routine application of DBS for the monitoring of drug resistance in individuals receiv-

Table 4. Summary of studies that evaluated the use of DPS/DSS for resistance genotyping

Reference	Year of publication	Laboratory methods								Test performance			Nucleic acid stability	Comment
		Specimen source	No.	Viral load range#	Subtypes	Filter paper	Spot volume (µl)	Drying time	Elution-extraction	Genotyping	Amplification success	Nucleotides*	Mutations*	Storage conditions	
Plantier et al. (35)	2005	DSS, ARV naïve and experienced, France	62	2.9 to 5.9	multiple	903	20	1h	QIAamp Viral RNA minikit (Qiagen)	Nested RT-PCR; PR 507 bp, RT 798 bp	82%; 86% 4-5 log10 cps/ml; 38% <4 log10 cps/ml	N/A	N/A	≤14 days at 4°C, no desiccant	
Dachraoui et al. (30)	2008	DPS, ARV naïve and experienced	33	3.1 to 5.9	B	903	20	1h	QIAamp Viral RNA minikit (Qiagen)	Nested RT-PCR; PR 507 bp, RT 798 bp	76%; 67% 4-5 log10 cps/ml; 0% <4 log10 cps/ml	N/A	N/A	9 days at room temperature	
Dachraoui et al (29)	2008	DPS and DSS	15	4.1 to 5.7	mostly B	903	20	1h	QIAamp Viral RNA minikit (Qiagen)	Nested RT-PCR; PR 507 bp, RT 798 bp	100% overall	N/A	N/A	2, 5, 7 day at room temperature	Different efficiency of 2 extraction methods

For each study a brief description is provided of laboratory methods used, DP/DSS test performance compared with the reference standard liquid plasma (unless stated otherwise), data on nucleic acid stability under environmental storage conditions, and any relevant comments. * Concordance with the reference standard liquid plasma; # HIV viral load expressed as $\log_{10}$ copies/ml. ARV, antiretroviral; bp, base pairs; NA, not available; PR, protease; RT, reverse transcriptase.

ing ART, particularly at the onset of treatment failure. The reduced assay sensitivities are mainly caused by the smaller specimen input volumes (20–200 µl), compared with liquid plasma specimens (140–500 µl) [29, 32, 35]. This results in an equivalently reduced number of HIV-1 RNA copies as input in the PCR reaction. Thus, further research should focus on improving the sensitivity of DBS assays, for instance by extracting virus from two or more spots [32, 33, 35] and by further concentration of the nucleic acid material upon extraction. Nested PCR has been used in several studies to increase sensitivity [27, 29–31, 33–38]. The main limitation for the analytical sensitivity of amplification assays based on dried fluid spots is caused by the amount of nucleic acid that is used as input in the first round PCR reaction. All investigations to improve the analytical sensitivity of amplification strategies based on dried fluid spots should therefore focus on optimization of extraction efficiency and to maximize the nucleic acid input in first round PCR reactions. A further downside of using nested PCR is the increased risk of carryover of amplicons, which poses a major limitation to its use particularly in low-resource settings.

Many researchers have reported high stability of nucleic acids (RNA and DNA) absorbed onto filter papers (mainly 903) and stored at ambient temperatures with humidity control. However, absence of desiccant [22, 31] and exposure to ambient and higher temperatures for extended periods [34] have been associated with degradation of nucleic acids. Most studies emphasize the importance of drying DBS completely prior to storage in a zip-locked plastic bag containing silica gel desiccant. It has been suggested that (partial) nucleic acid degradation might affect the longer DNA/RNA fragments that are required for sequencing to a greater extent than the shorter fragments amplified in viral load assays [28, 29, 31, 35]. Particularly, the large *pol* amplifications generated in various in-house or kit-based genotyping procedures might be more sensitive to nucleic acid degradation after storage under environmental conditions. The use of (in-house) RT-PCR assays that amplify a smaller genome fragment might result in higher amplification success rates [34, 37]. Overall, the results of molecular assays based on dried fluid spots are encouraging and support the use of dried fluid spots in areas where these materials can be (air)mailed to strategically situated reference testing facilities within a time span of a few days. For long-term DBS storage, the currently available data indicate that -20°C is preferable to preserve optimal amplification success.

Standard genotyping methods utilize only the plasma-derived virus population for amplification and sequencing. Unlike plasma, however, DBS contain proviral DNA archived in infected peripheral blood mononuclear cells (PBMCs). Although plasma RNA represents the population of short-lived actively replicating virus, proviral DNA from PBMCs is composed of a heterogeneous mix of DNA from acutely infected cells that actively produce virus as well as quiescent cells that comprise the viral reservoir [43, 44].

Resistance-associated mutations have been reported to emerge earlier in plasma than in the proviral archive in PBMCs [45], which might result in a higher sensitivity to detect early treatment failure in the plasma than in DBS. Notably, Steegen *et al*. [36] found that sequencing of proviral DNA from DBS resulted in failure of DBS to detect all mutations present in plasma, suggesting that in (early) treatment failure RNA sequencing is possibly superior to DNA sequencing. However, the extent of interference of proviral DNA sequences in the genotypic profiles generated from DBS might differ according to the disease stage, CD4+ T-cell counts, and treatment characteristics of the population as a result of the different dynamics of emergence and persistence of resistance mutations in plasma and PBMCs [46–48]. Further studies are warranted to elucidate the relative contribution of circulating RNA and proviral DNA in genotypic profiles generated from DBS in diverse populations [27, 33, 34].

The excision of individual spots from the filter papers prior to extraction is a labour-intensive procedure and carries risks for cross-contamination, which constitutes limitations for high-volume testing [49]. Therefore, the development of automated scissors or hole-punching machines and extraction methods are required to overcome these challenges. Logistically, the use of DPS involves the added step of venipuncture, requiring blood tubes containing EDTA and electricity-dependent centrifuge equipment, which might not always be available in remote areas. DBS would be the simplest method for blood sampling in remote low-resource areas, as it only requires a simple finger puncture, spotting and drying of a drop of whole blood on the filter paper, and (air)mailing it to a central laboratory. The need for a skilled phlebotomist and laboratory technician on-site as well as centrifuges, ultra-low-temperature freezers and dry ice for shipping can thus be avoided. However, the use of DBS might require active removal of the proviral cellular DNA [35], and an appropriate extraction method to remove PCR inhibitors present in erythrocytes [50].

Future studies should be directed towards further optimization and standardization of assay protocols, sensitivity and precision, nucleic acid stability under extreme storage conditions and, additionally for DPS, eliminating the need for on-site centrifugation to separate the plasma. Elimination of this step would broaden the applicability of DPS and render it suitable for use in settings that lack reliable electricity. Comparative studies of test performance of various commercial viral load assays are warranted. There is a need to coordinate and harmonize the research and development efforts on dried fluid spots conducted by various research groups. To this end, a global working group of international experts was recently established under coordination of WHO's Global HIV Drug Resistance Surveillance Network [42]. Multi-country collaborative studies have been initiated to refine technologies and definitely prove their utility for low-resource

Box. Recommendations for research
- Assess the performance of different extraction methods and viral load and resistance genotyping assays with dried fluid spots
- Develop optimized and standardized testing algorithms compatible with dried fluid spots
- Perform additional nucleic acid stability studies under extreme storage conditions (filter paper, storage and shipping temperature, humidity, time period, and UV light)
- Study interferences of proviral DNA in genotypes obtained from DBS in populations with diverse treatment characteristics
- Develop automated spot excision and nucleic acid extraction methods to enable high-volume testing
- Coordinate research efforts under guidance of global working groups of international experts

areas. Some recommendations for future research are listed in the Box. Finally, additional applications of the dried fluid spot technology should be considered; for instance, the study of the natural history of incident HIV-1 infection, therapeutic drug level monitoring and diagnosis and monitoring of relevant conditions such as hepatitis B and C.

In conclusion, the dried fluid spot filter paper technology offers the advantages of a stable specimen matrix, ease of sample collection and shipment with minimal biohazard risks, supporting its utility for the collection, storage and transport of large numbers of field specimens in low-resource settings. Available data have suggested that HIV-1 viral load determination and resistance genotyping from dried fluid spots is feasible. Although results to date are encouraging, assay sensitivities need to be improved, to allow application in regular monitoring of individual patients on ART. In addition, further nucleic acid stability studies as well as refinement and standardization of technologies are warranted to enable dried fluid spots to become the primary specimen collection device in resource-limited settings

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Contributors

RLH, RS and TFRdW designed the study. RLH and PWS performed the literature search, reviewed and extracted the data, and wrote the first draft of the manuscript. All authors contributed to subsequent drafts and reviewed the final manuscript.

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AFRIKA

Chapter 16

Global threat from drug resistant HIV in sub-Saharan Africa

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ABSTRACT

Roll-out of antiretroviral treatment for HIV in sub-Saharan Africa has been accompanied by rising rates of drug resistance. Raph Hamers and colleagues call for improved patient management and population based drug resistance surveillance to be integrated into national treatment programmes.

Since its introduction 16 years ago, combination antiretroviral therapy for HIV infection has saved millions of lives. In sub-Saharan Africa, the region with the highest HIV/AIDS burden, high level political commitment and substantial international funding have led to an unparalleled scale-up of access to treatment over the past eight years [1]. More than five million Africans infected with HIV are receiving antiretroviral therapy today—nearly half of those who are in immediate need [1]. However, little attention has been paid to the potential emergence and spread of drug resistant HIV and its public health implications. Drug resistant HIV variants selected for during treatment failure (acquired resistance) have the potential to limit the response to subsequent treatment and constitute a reservoir for onward transmission to newly infected individuals (transmitted resistance). Drug resistant HIV may severely restrict therapeutic options, and treatment costs will greatly increase when more people need second and third line antiretroviral regimens. It is therefore important for national HIV treatment programmes to monitor and manage mounting drug resistant HIV.

HIV DRUG RESISTANCE

In developed countries, management of combination antiretroviral therapy is based on individualised specialist care that includes frequent monitoring of plasma viral load to detect treatment failure, drug resistance testing to guide regimen choices, and a wide armamentarium of antiretroviral drugs (table 1) [2]. In Europe and North America, HIV sequential mono and dual therapies of nucleoside reverse transcriptase inhibitors (NRTIs), initially led to high levels of acquired and transmitted resistance [3-5] but careful management and use of more potent antiretroviral regimens have seen levels of transmitted resistance stabilising or declining [6-8].

By contrast, for resource limited countries the World Health Organization has developed a public health approach based on a decentralised service delivery, empirical first and second line antiretroviral regimens, and clinical or immunological definitions of treatment failure in the absence of monitoring of plasma viral load (table 1) [9]. Standard first line regimens include a dual NRTI backbone and a non-NRTI [9]. Second line regimens combine a ritonavir boosted protease inhibitor with two unused or recycled NRTIs [9], although availability may still be restricted [1].

Although the roll-out of antiretroviral treatment in sub-Saharan Africa used triple therapy from the onset, national health systems in many African countries have serious deficiencies that may exacerbate the development of drug resistance. These include the widespread use of low cost, substandard regimens (such as stavudine as part of first

Table 1. Differences in approaches to providing combination antiretroviral therapy between developing and developed countries

	Developing countries	Developed countries
Treatment model	WHO public health approach	Specialist driven, individualised patient management
Choice of regimen	WHO recommended empirical first line (2NRTIs+non-NRTI) and second line (bPI+2 unused/recycled NRTIs) therapies; restricted drug options, routine drug resistance testing not available.	Wide armamentarium of antiretroviral drugs Individualised therapies by routine use of drug resistance testing before starting or switching treatment
Therapeutic monitoring and diagnosis of treatment failure	WHO definitions of treatment failure using clinical criteria and, if available, CD4 cell counts. HIV viral load testing not generally available. Frequent unnecessary switching and prolonged failure	Close HIV viral load monitoring and timely regimen switching
Resources and infrastructure	Shortage of health professionals, limited training, deficient adherence counselling, inconsistent drug supply, weak enforcement of quality standards	Specialist care, intensive adherence counselling, continuous availability of drugs
Antiretroviral history	Roll-out since 2004-5 has used triple therapy	Widespread use of sequential mono and dual therapies before 1996
	Widespread use of single dose nevirapine for prevention of vertical HIV transmission	Since 1996, triple therapy, individualised regimens and close viral load monitoring

NRTI, nucleos(t)ide reverse-transcriptase inhibitor; bPI, ritonavir boosted protease inhibitor.

line treatment [10] and single dose nevirapine for prevention of mother to child HIV transmission); restricted access to monitoring of plasma viral load [11, 12]; treatment interruptions because of drugs supplies running out [13, 14]; suboptimal long term adherence [15]; and frequent drug-drug interactions (such as nevirapine with rifampicin in patients co-infected with tuberculosis) [16].

Recent studies in the region have reported increasing levels of transmitted drug resistance, mostly to non-NRTIs, as treatment has scaled up [17-19]. The rise in resistance to non-NRTIs is of particular concern because this drug class constitutes the foundation of current first line treatment regimens and prevention of mother to child transmission [9, 20]. The PASER-M study in six African countries estimated that the rate of transmitted drug resistance increases at 38% a year after roll-out of antiretroviral therapy [19], and that pre-therapy resistance more than doubles the risk of first line failure and of the further acquisition of drug resistance mutations in the first year of treatment [21]. Notably, we observed alarmingly high (9-12%) levels of transmitted drug resistance in Uganda [19], where antiretroviral treatment was introduced well ahead of nearby countries. With the establishment of the national HIV treatment programme and drug price reductions

in 2000, an estimated 10,000 patients were already receiving antiretroviral drugs by the end of 2002 [22], albeit with frequent interruptions for reasons of cost [14].

MONITORING VIRAL LOAD

An important challenge for antiretroviral programmes is how to identify patients in whom treatment is failing so that they can be switched promptly to second line therapy. Despite estimates that 10-24% of patients have detectable plasma viral load during first line therapy [21, 23], reported switching rates have been relatively low [1], partly because of the poor sensitivity of clinical and immunological criteria to detect therapy failure [12]. Additionally, the poor specificity of clinical and immunological criteria may lead to up to half of switches being unnecessary, which exhausts drug options and augments costs [12]. Given that the cost of second line drugs is more than double that of first line drugs [24], for every patient that is switched unnecessarily at least one patient will be held back from accessing life-saving antiretroviral treatment.

Although the benefits of routine monitoring plasma viral load in avoiding unnecessary switches [12] and accumulation of drug resistance [11, 12] are increasingly being acknowledged, its cost effectiveness in resource limited settings is still debated. Since any resources used for laboratory monitoring could divert funds away from expanding access to treatment, it is critical to establish optimal cost effective management. We recently established that routine monitoring of either CD4 cell counts or plasma viral load can save 15-30% of the cost of long term antiretroviral treatment in sub-Saharan Africa by averting the high costs of unnecessary switching to second line therapy [25]. Monitoring of plasma viral load has the added advantages of supporting adherence (as lapses are quickly apparent) [26] and identifying patients at risk of developing drug resistance [27] or transmitting HIV [28]. Studies in developed countries have suggested that there is limited benefit from continued measurements of CD4 cell counts in patients who have suppressed viral load [29, 30]. We therefore propose that antiretroviral programmes in sub-Saharan Africa should monitor plasma viral load rather than CD4 cell counts (once CD4 has risen above 200 cells/μL, when prophylaxis for opportunistic infections is no longer indicated). CD4 cell counts should be used only to establish eligibility for starting antiretroviral treatment and to determine the need for prophylaxis for opportunistic infections.

Scaling-up plasma viral load testing in Africa is feasible because recent technological advances have reduced test costs, simplified sample storage and shipment through the use of dried blood spots, and produced simpler and easy to maintain real-time poly-

merase chain reaction machines [31]. As test prices go down, the potential savings from laboratory monitoring will increase further.

ACCESS TO ALTERNATIVE DRUG REGIMENS

No matter how vigorous and successful the efforts to combat HIV drug resistance might be, given that the numbers of patients receiving antiretroviral drugs in sub-Saharan Africa are growing, increasing numbers will experience therapy failure. This necessitates improved access to alternative drugs with different modes of action and without cross resistance to NRTIs and non-NRTIs. Current WHO guidelines recommend ritonavir boosted atazanavir or liponavir as the preferred protease inhibitors for second line therapy [9]. Observational studies in Africa have shown that empirical boosted protease inhibitor based regimens can successfully resuppress HIV even in patients with extensive NRTI resistance [32, 33]. Clinical trials are underway to further assess the use of boosted protease inhibitors and integrase inhibitors in second line therapy and the potential for nucleoside sparing regimens. A recent meta-analysis suggested that failure of second line treatment was usually due to suboptimal adherence rather than development of resistance to protease inhibitors, which have a high genetic barrier to resistance [34]. Optimal long term support for adherence will therefore be critical because therapeutic options beyond second line regimens are prohibitively expensive for most African countries.

SURVEILLANCE OF DRUG RESISTANCE

To protect the sustained effectiveness of antiretroviral regimens, population based drug resistance assessment should be routinely integrated into the national HIV treatment programmes. Donors and technical agencies need to work with the national public health authorities to establish surveillance networks for tracking drug resistant HIV and sharing information with health professionals, policy makers, and researchers. WHO has initiated the Global HIV Drug Resistance Network (HIVResNet), which has developed a global strategy that aims to assess the emergence and transmission of drug resistance and to inform treatment guidelines [35]. More than 25 African countries have implemented one or more HIV drug resistance surveys [35], and the first WHO HIV drug resistance global report will be published in July. Additional initiatives, including the PharmAccess African Studies to Evaluate Resistance (PASER) network [36] and the Southern African Treatment and Resistance Network (SATuRN), have contributed by collecting resistance data, building laboratory capacity, and providing education. However, progress is being jeopardised by a decline in international donor support for surveillance.

QUALITY OF CARE AND UNIVERSAL ACCESS

A recent study estimated that spending $14.2bn during 2011-20 to keep HIV/AIDS patients alive is expected to save 18.5 million life years and yield as much as $34bn through increased labour productivity, averted orphan care, and deferred medical treatment for opportunistic infections and end of life care [37]. In addition to the large health gains, the economic benefits of antiretroviral treatment are likely to exceed programme costs within ten years.

Clearly, the strengthening of national HIV treatment programmes to expand access to treatment while minimising the development of resistance is a global priority. It is thus disappointing that the first casualty of the global financial crisis seems to have been the goal of universal access, with international funding agencies losing political will. The expenditure of the US President's Emergency Plan for AIDS Relief (PEPFAR) has levelled off since 2009 and the Global Fund to Fight AIDS, Tuberculosis, and Malaria has recently said it will fund only the continuation of essential prevention, treatment, and care services that are currently financed. This development will not only affect access but increase drug resistance through a rise in treatment interruptions, under-dosing, drug sharing, and the use of counterfeit drugs.

CONCLUSION

Rising drug resistant HIV in sub-Saharan Africa is a potential threat to the worldwide control of HIV/AIDS. National HIV treatment programmes should continue to expand access to antiretroviral drugs but also ensure quality in order to preserve treatment options for tomorrow. They need to ensure robust supply chains, improved diagnostic laboratory capacity, introduction of low cost viral load technologies, and the implementation of resistance surveillance (Box). Investment in such infrastructure now will be critical in the medium to long term.

Box. Steps to counter rising HIV drug resistance in sub-Saharan Africa
- Robust supply chains.
- Routine monitoring of viral load to ensure appropriate and timely switching.
- Access to second and third line drug regimens.
- Solid framework for surveillance of drug resistance.
- Continued international funding support to reach the goal of universal and sustainable access.

With declining international funding, the most efficient use of available resources will be critical. Mathematic modelling and economic analyses are needed to provide strategic

information to establish the optimal use of diagnostics and drugs and to determine funding priorities. There is no room for complacency. Without cumulative resistance surveillance data and commitment on the part of WHO, international funding agencies, and national governments to address programmatic challenges, emerging drug resistance has the potential to curb, and even reverse, further progress on breaking the HIV epidemic.

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Contributors

RLH wrote and revised the report. All authors contributed to intellectual content, helped to revise the report, and approved the final version. RLH is the guarantor.

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AFRIKA

Discussion


AFRIKA

Chapter 17

Summary and general discussion

Sub-Saharan Africa is the region most heavily affected by HIV/AIDS worldwide [1]. Access to combination antiretroviral therapy (ART) has rapidly expanded during the past decade to reach millions of HIV-1 infected people in the region [1], which has dramatically reduced HIV-related morbidity and mortality [2]. To allow the ART scale-up, the WHO-recommended public health approach has been critical. This approach is based on simplified treatment protocols, including standard first-line and second-line ART regimens, limited laboratory monitoring, and a decentralized service delivery [1]. Recommended first-line regimens combine a dual nucleos(t)ide reverse transcriptase inhibitor (NRTI) backbone with a non-NRTI (NNRTI). Recommended second-line regimens include two new or recycled NRTIs and a ritonavir-boosted protease inhibitor (bPI). Because of resource constraints, routine plasma viral load (pVL) monitoring to detect and manage ART failure is generally not feasible. Instead, ART failure is commonly diagnosed using WHO-defined clinical criteria and –if available– CD4 cell counts [1]. To date, relatively little attention has been paid to the potential emergence and spread of drug-resistant HIV-1 and its public health implications after the introduction of large-scale ART programs in the region.

Obstacles to universal and sustained ART access include weak ART program functioning, lack of sustainable long-term funding, and human resource constraints. An inevitable consequence of ART scale-up is treatment failure that selects for drug-resistant HIV-1 (acquired drug resistance). Such virus has the potential to limit the response to subsequent treatment and can be transmitted to newly infected individuals (transmitted drug resistance, TDR). Although the ART rollout in sub-Saharan Africa has employed (potent) triple combination therapy, the emergence of drug resistance could be exacerbated because of several reasons. Factors contributing to emerging drug resistance include suboptimal long-term adherence [3], lack of pVL monitoring [4, 5], treatment interruptions due to drug stockouts [6], drug-drug interactions [7] and the use of substandard regimens, including single-dose nevirapine for prevention of mother-to-child HIV transmission (PMTCT) [8]. Concern has been raised about rising drug-resistant HIV-1 in resource-limited countries as a potential threat to the worldwide control of HIV/AIDS [9].

To address the issue of HIV-1 drug resistance in resource-limited settings, a collaborative bi-regional program was established in the African and Asian-Pacific regions in 2006, entitled *Linking African and Asian Societies for an Enhanced Response to HIV/AIDS* (denoted LAASER). LAASER aimed to develop the regional capacities for the population-level assessment of acquired and transmitted HIV-1 resistance, funded by The Netherlands Ministry of Foreign Affairs in partnership with Stichting AidsFonds (2006-2011). As part of LAASER, the *PharmAccess African Studies to Evaluate Resistance* (PASER) network was established as a collaborative partnership of clinical sites, laboratories and research

groups in Kenya, Nigeria, South Africa, Uganda, Zambia, and Zimbabwe. To address the challenges associated with the high costs and complexity of HIV-1 genotypic resistance testing, PASER initiated a public–private consortium, called *Affordable Resistance Test for Africa* (ART-A), which aims to develop simplified, more affordable test algorithms for HIV-1 drug resistance.

This thesis is dedicated to clinical, epidemiological and public health studies related to HIV-1 drug resistance in sub-Saharan Africa, which were conducted as part of the PASER and ART-A programs. The aims of the studies described in the thesis were to define the epidemiology of TDR in HIV-1 infected populations in sub-Saharan Africa after the scale-up of ART, to assess the effects of pre-therapy resistance on the response to first-line or second-line ART, to assess patterns of drug-resistance mutations (DRMs) and their clinical impact in patients experiencing failure of standard first-line or second-line ART, and to explore the implications of emerging HIV-1 drug-resistance for public health policy in resource-limited countries.

As an introduction to the thesis, we reviewed the available data on HIV-1 drug resistance in sub-Saharan Africa before the start of the PhD research (before 2008) (**Chapter 2**), including an illustrative patient case study (**Chapter 3**). Early studies on TDR and acquired resistance are limited in number and quality, because of small and selected patient samples as well as heterogeneity across study designs, populations and time periods. In **Chapter 4**, we profiled the PASER-Monitoring (PASER-M) cohort. This multicentre observational cohort comprises a total of 2985 HIV-1 infected adults starting first-line or second-line ART, who were enrolled at 13 clinical sites in the abovementioned countries between March 2007 and September 2009 and followed up prospectively.

PART I: TRANSMITTED HIV-1 DRUG-RESISTANCE

In Europe and North America, HIV-1 drug-resistance has initially been driven by sequential non-potent mono and dual therapies of nucleoside reverse-transcriptase inhibitors (NRTIs) in the early days of HIV/AIDS treatment, leading to high levels of acquired resistance in treated individuals in the 1990s. This was associated with high levels of TDR, peaking in some settings at over 20% before levelling off at between 9-15% more recently [10-13]. Recent stabilizing or declining levels of TDR in resource-rich countries are likely attributed to declining incidence of acquired resistance, due to the use of more potent ART regimens, regimen individualization by use of pre-therapy resistance testing, and close pVL monitoring [14]. It has been difficult to predict TDR trends for sub-Saharan Africa after the ART scale-up based on the experiences from resource-rich

countries, given that the histories of antiretroviral drug access and the health system conditions differ significantly between these settings [15-17]. Mathematical modelling of TDR in Africa, attempted to inform public-health strategies, has yielded conflicting results. For instance, one study predicted low rates of TDR (<5%) until 2015 [18], whilst another study, based on the Botswana ART program, predicted that TDR could reach 15% by 2009 if acquired resistance rates were high [19, 20]. These discordant findings highlight the importance of timely and accurate empirical data.

In **Chapter 5**, we compared pre-therapy drug resistance between antiretroviral-naïve (n=523) and antiretroviral-exposed (n=25) persons, who were about to start standard first-line ART at either of three clinical sites in Lusaka, Zambia [21]. 5% of the study population reported previous antiretroviral exposure, either as ART, single-dose nevirapine for PMTCT, combination therapy for PMTCT, or unspecified. The main finding was that drug-resistant HIV-1 was detected in 16.0% of patients who were antiretroviral-exposed, compared with 5.2% of antiretroviral-naïve patients (p=0.022). Pre-therapy DRM patterns and frequencies did not differ between an established private versus two recently introduced free ART programs.

In **Chapter 6**, we reported that, overall, 5.6% of 2436 antiretroviral-naïve individuals from 11 areas in Kenya, Nigeria, South Africa, Uganda, Zambia and Zimbabwe harboured TDR, which was mostly NNRTI-associated and limited to a single drug-class [22]. The prevalence of TDR in antiretroviral-naive individuals appeared substantially higher in Uganda (12%), where antiretroviral drugs were available at least five years ahead of the nearby countries, compared with the other five countries (<5%). TDR was estimated to increase with time since the start of the ART roll-out, by –on average– 38% (95%CI 13-68; p=0.01) per year. For NNRTI-resistance, the increase was estimated at 35% (95%CI 1-81; p=0.041) per year.

In **Chapter 7**, we reported a TDR prevalence of 8.6% (95%CI 3.2–17.7) among 70 recently HIV-1 infected attendees at voluntary counseling and testing sites in Kampala, Uganda, in 2009, which was ten years after the local ART roll-out [23]. This estimate was likely to represent an increase compared to the previous survey in 2006–2007 that did not detect any DRMs among 47 recently infected pregnant women [24]. This study was among the first to suggest an increase in TDR between repeated surveys within the same geographic area in Africa, although the subsequent surveys targeted different subpopulations.

The PASER-M cohort is one of the largest studies on the topic of HIV-1 drug-resistance to date. An important strength of the study was the large number of patients and sites participating, representing a diverse spectrum of patient populations, clinic types, ART

regimens and HIV-1 subtypes. However, although the PASER network was designed to geographically represent the African sub-regions, the generalizability of the study was limited by the fact that only six countries were included and that the collaborating clinical sites were not selected randomly. To enable a more comprehensive description of regional TDR time trends, PASER has collaborated in a WHO-initiated meta-analysis of all available TDR studies [25]. Of the 218 datasets comprising 26,102 untreated patients from 42 countries, PASER accounted for 9% of all patients and 17% of patients from sub-Saharan Africa. The meta-analysis demonstrated an increasing prevalence of TDR in the east and southern African sub-regions. East Africa had the highest estimated rate of increase at 29% per year (95%CI 15-45, p=0.0001) since roll-out, with an estimated prevalence of TDR at eight years post-roll-out (circa 2011) of 7.4% (95%CI 4.3-12.7). The increase in southern and West/Central Africa was 14% (95%CI 0-29, p=0.054) and 3% (95%CI -0.9-16, p=0.618) per year respectively. There were substantial increases in NNRTI resistance in east Africa [36% per year (95%CI 21-52, p<0.0001)] and southern Africa [(23% per year, 95%CI 7-42%, p=0.0049)]. Rising TDR levels have also been reported in recent studies among newly infected populations from east [26], central [27] and southern Africa [26]. These data corroborate the PASER findings reported in **Chapters 6 and 7**, adding to the increasingly convincing evidence that the scale-up of ART in Africa is driving a rise in TDR, particularly associated with NNRTIs. The rise in NNRTI resistance is of particular concern, as this drug class constitutes the foundation of currently recommended first-line ART and PMTCT regimens [28, 29]. Repeated surveys in the same and other settings and subpopulations are urgently needed to evaluate the evolution of TDR over time.

There is ongoing debate on what is the most appropriate target population to conduct TDR surveys, i.e. either recently infected persons or pre-therapy patients at ART initiation. Recently infected populations would maximize the detection of true TDR mutations, and minimize the contribution of acquired resistance due to prior antiretroviral exposure, thus enabling reliable analyses of TDR time trends. However, there are significant challenges in identifying individuals during recent HIV-1 infection. For instance, TDR surveys among newly diagnosed populations at antenatal care clinics or voluntary counseling and testing sites –who are likely to be recently infected– are logistically complex and require a high screen load, thus limiting their feasibility especially in low-prevalence settings. This has also been our experience in conducting the PASER-S surveys. By contrast, in pre-therapy populations (such as in PASER-M), who are easy to sample at ART sites, the prevalence of true TDR may be underestimated due to reversion [30], and TDR reflects past ART availability at the population-level. Moreover, there is a risk of contributing acquired resistance due to (undisclosed) prior antiretroviral exposure. Nevertheless, from a public health perspective, it must be recognized that pre-therapy

resistance, whether due to transmission or undisclosed prior antiretroviral exposure, will both impact adversely on the response to national ART programs. Furthermore, TDR survey results from pre-therapy populations will be directly applicable to inform current ART guidelines. Therefore, given the abovementioned feasibility issues and the restricted resources that are currently available for HIV-1 drug resistance surveillance, we here argue that surveying pre-therapy resistance in populations at ART initiation should be prioritized. Where feasible, surveying TDR in newly infected populations, if conducted sequentially, will provide important additional information to guide drug-resistance prevention strategies in ART programs and to inform future ART guidelines.

PART II: ANTIRETROVIRAL TREATMENT AND ACQUIRED DRUG-RESISTANCE

In Europe and North America, pre-therapy resistance testing is routinely performed to guide first-line ART choices [31, 32], which has been shown to mitigate virological failure in persons with TDR [33]. By contrast, in resource-limited countries, drug resistance testing is not routinely available and WHO-recommended first-line and second-line ART regimens are empirically prescribed. In **Chapter 8**, we reported that among 2733 PASER-M participants who had received one year of standard first-line NNRTI-based ART, the presence of pre-therapy resistance more than doubled the risk of virological failure and the further acquisition of DRMs, in those patients who received partly-active regimens — i.e., that included at least one drug to which the virus had reduced susceptibility [34]. These findings were largely in agreement with results from a large collaborative analysis in Europe [33]. Additionally, 70% of participants who had pre-therapy resistance were empirically started on a suboptimum first-line regimen — comprising nearly 5% of the total study population [34]. Given the low genetic barrier of NNRTI-based regimens, our study emphasized the need for at least three fully-active antiretroviral drugs in first-line regimens to ensure an optimum virological response and to prevent the further acquisition of resistance. Independently of pre-therapy resistance, we found that previous use of ART or PMTCT, failure to achieve an increase in CD4 cell count in the first six months of ART, and prolonged non-adherence below 95% were associated with virological failure and the further acquisition of resistance.

There is limited knowledge of DRMs in HIV-1 non-B subtypes and their clinical relevance, despite the fact that more than 90% of HIV-1 infections globally belong to non-B subtype variants [35]. In **Chapter 10**, we observed that among 250 patients at time of switch after prolonged first-line failure, in the absence of pVL monitoring, 88% had at least one DRM, including high frequencies of M184V and accumulated NRTI and NNRTI mutations [5]. Several studies in patients with late detection of ART failure have reported high fre-

quencies of accumulated NRTI and NNRTI mutations, including TAMs, K65R and Q151M, conferring broad cross-resistance [4, 5, 36-38]. In **Chapter 9**, we observed that among 142 patients who experienced virological failure after the first year of ART, 70% had at least one DRM, mostly M184V and NNRTI-associated [39]. Observed DRM patterns in both studies were more extensive than for cohorts that received close (three-monthly) pVL monitoring in South Africa [40] and resource-rich countries [41, 42]. Thus, these recent observational studies from the region corroborate the notion that routine pVL monitoring prevents accumulation of DRMs and preserves HIV susceptibility.

Recent studies have suggested increased rates of the K65R mutational pattern in subtype C, compared with subtype B, which may be due to the nature of the subtype C RNA template of the viral reverse transcriptase [43, 44]. By contrast, in subtype B, the generation of D67N and TAMs is facilitated instead of K65R [43, 44]. In our multi-country African study of mostly subtype A, C, or D infected persons, the K65R pattern was frequently selected after failure on stavudine (15%) or tenofovir (28%) containing first-line regimens (**Chapter 9**) [39]. This finding suggested that, after failure of a stavudine-containing first-line regimen, zidovudine rather than tenofovir (as recommended by the current WHO guidelines [28]), might be the preferred second-line NRTI in non-B subtype infected populations where stringent pVL monitoring is lacking. The data reported in **Chapters 9 and 10** also suggested that the second-generation NNRTIs etravirine or rilpivirine are unlikely to be effective as part of second-line ART, if combined with two NRTIs, given the high frequencies of Y181C and accumulated NNRTI mutations.

Few data are available on the extent of misdiagnosis of true virological failure using the common diagnostic approach that adopts clinical and/or immunological definitions of ART failure [45, 46]. In **Chapter 10**, we demonstrated that, in the absence of pVL testing, switches from first- to second-line ART occur unnecessarily in up to 50% of cases [5]. Assuming that second-line treatment is 2.3-fold more expensive than first-line (CHAI prices [47]), this means that for every patient who is switched to second-line unnecessarily, at least one other patient will be held back from accessing first-line ART. To further investigate this important finding, we conducted a cost-effectiveness analysis that attempted to quantify and compare the economic implications of different diagnostic strategies (**Chapter 14**).

In **Chapter 11**, we investigated the initial response to empiric second-line ART in 243 patients who experienced first-line failure [48]. In our cohort, we found that the risk of second-line ART failure was not increased in participants who carried a virus with predicted reduced susceptibility to at least one prescribed second-line drug, compared with those who received ART that was predicted to be fully-active. In addition to our study,

one study in Malawi has suggested that empiric bPI-based regimens can successfully re-suppress HIV-1 replication (in 85-86% of patients), despite the presence of extensive NRTI resistance [48, 49]. By contrast, the ACTG5230 trial, evaluating bPI monotherapy after first-line failure, found that about one third of patients who achieved <400 HIV-RNA c/ml after 24 weeks, appeared to have incomplete viral suppression at between 40-200 HIV-RNA c/ml [50]. Additionally, the randomized HIV Star Study in Thailand found that second-line bPI monotherapy was virologically inferior to triple therapy in patients failing NNRTI-based first-line (61% vs 83% HIV-RNA<50 c/ml) [51]. Thus, bPI monotherapy should not currently be recommended as second-line therapy, and large international trials are underway to further assess this issue.

A recent systematic review and meta-analysis of outcomes of patients on second-line ART in resource-limited settings showed that rates of virological failure are high (23%, 27% and 38% at 12, 24 and 36 months, respectively) and associated with duration of exposure to previous drug regimens and poor adherence, rather than resistance development to bPIs, which is likely attributable to their high genetic barrier to resistance [52]. Therefore, a major concern seems to be poor long-term adherence, especially given that therapeutic options beyond second-line are very expensive and largely non-existent in most African countries.

PART III: PUBLIC HEALTH POLICY

An effective public health framework is required to assess and contain HIV-1 drug-resistance in sub-Saharan Africa [53]. In **Chapter 12**, we reported practice-based lessons learned in the PASER network [54]. Through the assessment of resistance at site and regional levels, PASER has contributed to evidence-based recommendations to inform ART guidelines and to provide feedback on the effectiveness of HIV-1 treatment and prevention programs. The PASER network has contributed to the goals of the WHO Global HIV Drug Resistance Network (HIVResNet) [53]. The sustainability of the PASER network is challenged by funding limitations, the need for continued training and education, constraints in human resources, a persistently vulnerable general health infrastructure, and the urgent need for simplified and affordable diagnostic technology.

WHO recommends that ART programmatic factors, such as prescribing practices, patient retention and drug supply, which are associated with acquired drug resistance, are monitored to optimize the quality of patient care [53]. The minimum-resource WHO-defined early warning indicators (EWIs) make use of data that are routinely collected in patients' medical and pharmacy records. In **Chapter 13**, we reported the assessment

of the EWIs in the PASER network from 2007-2009. Eleven of 13 (85%) sites prescribed appropriate first-line ART regimens for all patients; 12 (92%) sites met the targets of ≤20% loss to follow-up and ≥70% pVL suppression; all sites achieved ≥70% retention on first-line ART. EWI assessment in the PASER network identified vulnerable aspects of ART programs and triggered programmatic interventions aimed at minimizing resistance development. Interestingly, a comprehensive WHO assessment of 907 ART programs in the region between 2004 and 2009 documented drug stock-outs in about 40% of sites, more than 20% of loss to follow-up in 40% of sites, and ART prescription congruent with national guidelines to 100% of patients in 74% of sites [55]. Important gaps in service delivery and program performance affect a considerable proportion of ART programs, particularly with respect to the fragility of procurement and supply systems and inadequate patient retention.

Previous evaluations have yielded conflicting results regarding the survival gains and cost-effectiveness associated with ART diagnostic strategies based on CD4 cell counts alone, or CD4 cell counts combined with pVL monitoring [56-58]. The benefits of routine pVL monitoring in avoiding unnecessary switches and resistance accumulation are increasingly being acknowledged (**Chapter 10**) [5]. Since any resources used to conduct laboratory testing could divert funds away from expanding access to ART, it is critical to establish the most cost-effective ART management strategies. In **Chapter 14**, we reported a Markov-based cost-effectiveness analysis, establishing that laboratory-based diagnostic strategies, using either CD4 cell counts or pVL, can provide substantial (15-30%) cost savings for long-term ART management in sub-Saharan Africa by averting the high costs of unnecessary switching to second-line therapy [59]. This model is the first cost-effectiveness analysis we know of to compare different diagnostic strategies for ART management that includes a "pVL only" strategy, without concomitant CD4 cell counts. pVL monitoring has the public health advantages of supporting adherence [60], and identifying patients at risk of developing resistance [61] or transmitting HIV [62]. Previous studies in developed countries have suggested that there is limited benefit from continued measurements of CD4 cell counts in patients who have achieved pVL suppression [63, 64]. Use of CD4 counts could thus be restricted to establish eligibility for ART initiation, and to determine the need for prophylaxis for opportunistic infections. Challenges to scaling-up pVL testing in Africa are surmountable, since recent technological advances enable lower test cost, simplified sample storage and shipment with the use of dried blood spots, as well as easy-to-maintain real-time PCR machines [65]. As the number of persons receiving ART rises and test prices go down, the potential health benefit and cost savings from the use of laboratory monitoring will further increase.

Dried spots on filter paper made of whole blood (dried blood spots; DBS), plasma (dried plasma spots; DPS) or serum hold promise as an economical and practical alternative specimen source to liquid plasma for pVL determination and drug resistance genotyping in resource-limited countries. In **Chapter 15**, we reviewed the evidence that was available up to 2009 for the utility of dried fluid spots for the determination of pVL and resistance genotyping. Available data indicated that pVL determination and resistance genotyping from DBS and DPS is feasible. Limitations included reduced analytical sensitivity resulting from small analyte volumes, nucleic acid degradation under environmental conditions, impaired efficiency of nucleic acid extraction, potential interference of archived proviral DNA in genotypes obtained from DBS and the excision of spots from the filters in high-volume testing. The current sensitivity in resistance testing is probably appropriate for public health surveillance among pre-therapy populations. The ART-A consortium and other groups are involved in ongoing research that aims to improve analytical sensitivity and assay conditions, in order to expand the routine application of DBS in public health surveillance as well as the therapeutic monitoring of individuals receiving ART.

In **Chapter 16**, we expounded a viewpoint that rising drug-resistant HIV-1 in sub-Saharan Africa is a potential threat to the worldwide control of HIV/AIDS. The highest priority remains achieving the goal of providing ART to 15 million people by 2015 worldwide. In addition to large health gains, the economic benefits of ART have been estimated to exceed program costs within ten years of investment [66]. The strengthening of national HIV treatment programs that include robust supply chains, improved access to (low-cost) pVL technologies, improved access to second and third-line regimens, and a population-based framework for resistance assessment are a global priority. Investment in such infrastructure now will be worthwhile in the medium to long term.

FUTURE PERSPECTIVES

Continued and increased international funding support remains essential to reach the goal of universal access and to improve the quality of HIV/AIDS treatment in sub-Saharan Africa. PASER, in conjunction with other studies, has provided compelling evidence that HIV-1 drug-resistance is emerging after the ART scale-up in sub-Saharan Africa, which represents a potential threat to the worldwide control of HIV/AIDS. To ensure continued effectiveness of HIV/AIDS treatment, ART guidelines in resource-limited countries should take into account the most recently available local, regional and global data on HIV-1 drug resistance.

There is ongoing debate on what should be the public health response to high levels of TDR in any given setting. There is not a clear-cut TDR level at which policy change is indicated in all settings. Potential options for public health interventions include a shift in standard first-line ART from NNRTI-based to bPI-based, the introduction of individual-level drug resistance testing before ART initiation, and the introduction of pVL monitoring for early failure detection. A shift to bPI-based ART as standard first-line therapy at current drug prices is generally considered the last resort by many experts, given that such a change would have major programmatic implications, substantially increase drug cost and seriously restrict options for constructing an effective second-line regimen with currently available drugs. Of note, the 2011 Clinton HIV/AIDS Foundation (CHAI) drug prices are US$169 per year for a recommended first-line regimen (tenofovir, lamivudine, efavirenz) and US$395 per year for a second-line regimen (atazanavir/ritonavir, tenofovir, lamivudine), which is a 2.3-fold cost difference [47]. Routine individualized pre-therapy resistance testing is not likely to become feasible for most ART programs in the region, because of serious constraints in laboratory capacity and financial resources. Implementing routine pVL monitoring seems a more feasible option. The accurate identification of patients who experience virological failure will preserve drug options by avoiding the incremental cost associated with unnecessary switching and by reducing drug resistance accumulation. Moreover, a recent model of HIV transmission predicted that TDR in resource-limited settings will be reduced if some form of pVL monitoring is introduced [67]. Mathematic modeling and economic analyses will be crucial to provide strategic information in establishing the most cost-effective use of diagnostic strategies and drugs and to determine funding priorities. In this respect, it should be noted that the level of TDR is only one factor in determining whether a policy change for ART programs will be cost-effective. Finally, based on our study findings, we can make the pragmatic recommendation that the accurate, routine screening of previous exposure to ART and PMTCT should be strengthened, and that for individuals who report previous use of (NNRTI-based) ART or PMTCT, bPI-based first-line ART, or at least intensified monitoring, should be considered.

Robust ART programmatic evaluation of site-level factors associated with acquired resistance can play an important role in identifying and addressing deficiencies in ART delivery. The WHO-defined EWIs should become integrated into routine ART program monitoring and evaluation systems. To make this feasible, the currently recommended set of EWI will need to be simplified and synchronized with existing indicators. Additionally, repeated population-level laboratory-based drug resistance surveys are imperative and should be routinely integrated in national HIV treatment programs. Extended capacity for quality-assured drug resistance testing is needed to facilitate the conduct of these surveys. Funders and national governments must step up to support and sustain

population-based drug resistance surveillance. Evidence-based information will serve as a powerful advocacy tool towards funding agencies and policy makers to advance the sustainability and quality of HIV/AIDS treatment.

Further research is needed to investigate optimal strategies to prolong the effective use of first-line ART regimens, and to investigate in what conditions bPI-based regimens may offer a feasible alternative to current first-line regimens in view of increasing TDR levels. Optimal strategies for ART sequencing need to be determined, including the long-term effectiveness of bPI-monotherapy, the impact of multidrug NRTI mutations on empiric bPI-based second-line regimens, and the possible role of new drug classes (e.g. integrase inhibitors, second-generation NNRTIs). Additionally, it needs to be established what is the most cost-effective pVL threshold for switching therapy, and what is the role of resistance genotyping in resource-limited countries.

Because of clinical benefits, international guidelines recommend earlier initiation of ART at CD4 <350 or even <500 cells/μl [28, 31]. As of recent, early treatment has attracted significant attention as a promising tool to reduce the number of people acquiring HIV infection [62]. Little is yet known on what will be the population effects of the widespread implementation of early treatment in sub-Saharan Africa, in terms of HIV prevention, survival and drug resistance development. Earlier ART initiation could be anticipated to lead to a further rise in TDR. It needs to be established whether this risk is outweighed by a reduction in the number of new HIV infections.

CONCLUSION

The introduction of large-scale HIV/AIDS treatment in sub-Saharan Africa less than a decade ago has saved millions of lives. There is now compelling evidence from PASER and other studies that HIV-1 drug-resistance is on the rise after the ART scale-up, which may restrict therapeutic options, increase mortality, and augment treatment costs. To date, substantial progress has been made in assessing the development and spread of drug-resistant HIV-1 and its potential public health implications. Concerted action by international agencies, national governments, ART programs and major funding agencies will be critical to identify and address programmatic challenges associated with drug resistance, and preserve the long-term effectiveness of available ART regimens in Africa. Greater funding, political will and infrastructure are required to sustain and expand global resistance surveillance efforts, in order to ensure responsible provision of life-long HIV/AIDS treatment. WHO, in conjunction with experts in the field, should step up in convincing the decision-makers in governments and funders of the urgency of

HIV-1 drug resistance as a possible threat to the success of the global HIV/AIDS control. Without continued and increased international efforts and funding support, emerging resistance has the potential to curb, and even reverse, further progress on breaking the HIV epidemic.

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AFRIKA

Addendum

Samenvatting (Summary in Dutch)

HIV/AIDSBEHANDELING IN AFRIKA

Afrika ten zuiden van de Sahara wordt wereldwijd het hardst getroffen door het humane immunodeficiëntievirus (hiv), de veroorzaker van aids (*acquired immunodeficiency syndrome*). Wereldwijd zijn er naar schatting 34 miljoen mensen met hiv geïnfecteerd, van wie bijna 23 miljoen in Afrika. Aids is het gevolg van een sterk verzwakt afweersysteem doordat het virus een belangrijke groep witte bloedcellen, de *CD4+ lymfocyten*, infecteert en uitschakelt. Hiv-behandeling met een combinatie van tenminste drie hivremmers, de zogenaamde antiretrovirale combinatietherapie (ART), zorgt ervoor dat de virusvermenigvuldiging wordt onderdrukt waardoor het immuunsysteem zich kan herstellen. Sinds 1996 heeft ART het welzijn en de levensverwachting van mensen met hiv/aids spectaculair verbeterd. Sociale mobilisatie, sterke politieke wil en aanzienlijke internationale donorsteun hebben ertoe geleid dat in het voorbije decennium hiv-behandeling op grote schaal beschikbaar is gekomen in Afrika. Dit is één van de grootste successen in de geschiedenis van de moderne geneeskunde. Naar schatting hebben nu vijf miljoen hiv-geïnfecteerde Afrikanen toegang tot levensreddende behandeling. Dit is nog steeds pas de helft van alle mensen die de behandeling urgent nodig hebben.

Bij gebrek aan financiële middelen, mankracht en infrastructuur, vindt hiv-behandeling in ontwikkelingslanden plaats volgens vereenvoudigde richtlijnen, met gestandaardiseerde eerstelijns en tweedelijns medicijncombinaties en slechts beperkte monitoring van de therapie. Dit is in tegenstelling tot de situatie in ontwikkelde landen, waar regelmatige controle van de *virale lading* in het bloed wordt gebruikt om te beoordelen of de behandeling nog effectief is, en waar de keuze van de optimale medicijncombinatie wordt bepaald op geleide van een hiv-resistentietest. De standaard combinatietherapie in ontwikkelingslanden bestaat uit een eerstelijn van twee zogeheten *nucleoside reversetranscriptaseremmers* (NRTI) en een *non-NRTI* (NNRTI), en –als de eerstelijn faalt– een tweedelijn van twee NRTIs en een *ritonavir-boosted proteaseremmer* (bPI).

THERAPIERESISTENTIE

Gezien de snelle toename van het aantal hiv-patiënten in Afrika op behandeling, zullen er naar verwachting ook steeds meer mensen op een zeker moment *therapiefalen*

ontwikkelen. Therapiefalen kan leiden tot het ontstaan van mutaties in het virus die de gevoeligheid voor het medicijn verminderen. Dit fenomeen wordt *resistentie* genoemd. De kans op het ontstaan van resistente hiv-varianten is bij nauwkeurig en consequent gebruik van de combinatietherapie sterk verminderd, maar omdat hiv een virus is dat snel deelt en muteert blijft het risico van resistentieontwikkeling (*verworven* of *secundaire* resistentie) bestaan. Andere personen kunnen op hun beurt worden geïnfecteerd met een dergelijk resistent virus, wat *overgedragen* of *primaire* resistentie wordt genoemd. Hiv-resistentie kan snel de kop opsteken als gezondheidszorgsystemen slecht functioneren, voornamelijk als gevolg van therapieonderbrekingen door bevoorradingsproblemen, het niet consequent innemen van alle pillen, en het te laat op het spoor komen van therapiefalen, omdat de test om de viruslading in het bloed te meten veelal niet voorhanden is. De mogelijke opkomst en verspreiding van hiv-resistentie in Afrika heeft vooralsnog weinig aandacht gekregen.

DOELSTELLINGEN PROEFSCHRIFT

Dit proefschrift betreft onderzoek naar klinische, epidemiologische en volksgezondheidsaspecten van hiv-resistentie in Afrika. De doelstellingen zijn het beschrijven van de epidemiologie van primaire resistentie na de snelle uitbreiding van hiv-behandeling, het bestuderen van de effecten van pre-therapie resistentie op de effectiviteit van eerstelijns- en tweedelijnsbehandeling, en het bestuderen van de resistentiepatronen in patiënten met therapiefalen van de eerstelijn of tweedelijn. De studies beschreven in dit proefschrift zijn uitgevoerd in een nieuw surveillancenetwerk in Afrika (*PharmAccess African Studies to Evaluate Resistance*, PASER), dat zich ten doel stelt om de regionale kennis- en capaciteitsinfrastructuur te verbeteren op het gebied van hiv-resistentie, samen met haar zusternetwerk in Azië. PASER bestaat uit hiv-behandelklinieken, medische laboratoria en onderzoeksgroepen in Kenia, Nigeria, Oeganda, Zambia, Zimbabwe en Zuid-Afrika.

Als inleiding geven we een overzicht van de hiv-resistentiegegevens in Afrika die voorhanden waren voor de start van het promotieonderzoek (voor 2008) (**Hoofdstuk 2**), inclusief een illustratieve patiëntcasus (**Hoofdstuk 3**). In **Hoofdstuk 4** introduceren we het PASER-Monitoring (PASER-M) cohort, bestaande uit 2985 hiv-geïnfecteerde volwassenen die met eerstelijns of tweedelijns behandeling startten tussen maart 2007 en september 2009 in 13 klinieken in de bovengenoemde zes landen.

RESULTATEN BESCHREVEN IN DIT PROEFSCHRIFT

Deel I: Primaire HIV resistentie

In **Hoofdstuk 5** beschrijven we dat bij hiv-geïnfecteerde mensen in Lusaka, Zambia, vóór het starten van eerstelijns ART, hiv-resistentie aanwezig was bij slechts 5% van hen die nog nooit een hiv-remmer hadden gebruikt. Echter, van de mensen die al eerder hiv-remmers ter behandeling of profylaxe hadden gebruikt, had al 16% een resistent virus.

In **Hoofdstuk 6** beschrijven we dat primaire resistentie, en dan vooral NNRTI-resistentie, werd gevonden bij 5.6% van 2436 onbehandelde hiv-geïnfecteerde personen afkomstig uit 11 gebieden in Kenia, Nigeria, Zuid-Afrika, Oeganda, Zambia en Zimbabwe. De prevalentie van primaire resistentie bleek aanzienlijk hoger in Oeganda (12%), waar hiv-remmers tenminste vijf jaar eerder dan in omliggende landen beschikbaar kwamen, dan in de andere vijf landen (<5%). Onze schatting is dat primaire resistentie gemiddeld per jaar met 38% (35% specifiek voor NNRTI-resistentie) toeneemt in deze groep patiënten na het lokaal beschikbaar komen van hiv-behandeling.

In **Hoofdstuk 7** rapporteren we dat, tien jaar na het beschikbaar komen van hiv-behandeling in Kampala, Oeganda, er één van iedere elf mensen die een nieuwe hiv-infectie oplopen geïnfecteerd wordt met een resistent virus. Deze studie was een van de eerste die een toename van primaire resistentie liet zien tussen herhaalde prevalentiemetingen (0% in 2006-2007) binnen eenzelfde gebied in Afrika.

Een meta-analyse van alle tot nu toe beschikbare gegevens over primaire resistentie (totaal 26102 onbehandelde personen uit 42 landen), inclusief die van PASER (9% van het totaal en 17% van alle Afrikanen), laat zien dat primaire resistentie een groeiend probleem is in Oost- en Zuidelijk Afrika. De toename van NNRTI-resistentie is van bijzonder belang, omdat deze groep medicijnen de hoeksteen is van de huidige eerstelijns therapie.

Deel II: Antiretrovirale therapie en secundaire HIV resistentie

In **Hoofdstuk 8** laten we zien dat de aanwezigheid van pre-therapie resistentie tegen (een of meer medicijnen van) de standaard eerstelijn de kans op therapiefalen meer dan verdubbelt en leidt tot meer resistentiemutaties. Dientengevolge kreeg 70% van de studiedeelnemers bij wie pre-therapie resistentie was vastgesteld (dat wil zeggen 5% van de gehele studiepopulatie) een suboptimale eerstelijns therapie. Deze bevindingen benadrukken dat het van groot belang is dat de eerstelijn tenminste drie werkzame medicijnen bevat.

We rapporteren hoge frequenties van accumulatie van NRTI- en NNRTI-resistentiemutaties bij patiënten met langdurig therapiefalen (**Hoofdstuk 10**), terwijl we bij patiënten met kortdurend therapiefalen beduidend minder complexe resistentiepatronen waarnamen (**Hoofdstuk 9**). Onze observationele studies laten zien dat frequente monitoring van de viruslading de accumulatie van resistentiemutaties kan beperken en daarmee de gevoeligheid van hiv voor de standaardtherapieën behoudt. We gaan in **Hoofdstuk 10** verder in op de gevolgen van de wijze waarop de effectiviteit van de behandeling wordt geëvalueerd. Bij patiënten van wie de behandelcombinatie werd gewijzigd (*geswitcht*) van eerstelijn naar tweedelijn vanwege het vermoeden van therapiefalen op basis van alleen de klinische symptomen en het CD4-getal (en niet de viruslading in bloed) bleek deze switch bij ongeveer de helft onnodig te zijn (want de virusreplicatie was onderdrukt). Dit leidt tot kostenstijging, meer bijwerkingen en minder behandelopties (zie ook **Hoofdstuk 14**).

In **Hoofdstuk 11** laten we zien dat *empirische* tweedelijns therapie bestaande uit een bPI en twee NRTIs effectief de virusreplicatie kan onderdrukken, zelfs in aanwezigheid van uitgebreide NRTI-resistentie.

Deel III: Volksgezondheid

Hoofdstuk 12 beschrijft relevante praktijkervaringen in de ontwikkelingsfase van het PASER-netwerk. De duurzaamheid van het PASER-netwerk staat onder druk door beperkte financiële middelen, de noodzaak tot voortdurende educatie, kwetsbare zorgsystemen en een dringende behoefte aan vereenvoudigde en goedkopere diagnostische testen.

In **Hoofdstuk 13** rapporteren we de evaluatie van de zogeheten *Early Warning Indicators (EWI)* voor hiv-resistentie, ontwikkeld door de Wereldgezondheidsorganisatie, in het PASER netwerk (2007-2010). Bij de EWIs wordt gebruik gemaakt van routinematig verzamelde patiëntgegevens per kliniek die zijn geassocieerd met hiv-resistentie. Bijvoorbeeld, 11 van de 13 klinieken schreven correcte eerstelijnscombinaties voor aan alle patiënten; 12 van de 13 klinieken haalden de doelstelling wat betreft patiëntretentie (meer dan 80% van de patiënten) en virusonderdrukking (meer dan 70% van de patienten) in het eerste behandeljaar. Negen van de dertien klinieken voldeden niet aan de doelstelling voor één or meer van de indicatoren. Door kwetsbaarheden bloot te leggen in het functioneren van behandelprogramma's, kan de kwaliteit van zorg worden verbeterd.

Hoofdstuk 14 is een kosteneffectiviteitsanalyse van verschillende strategieën van therapiemonitoring. We tonen aan dat monitoring op basis van alleen de viruslading

(zonder het CD4-getal) belangrijke (15-30%) kostenbesparingen kan opleveren, ten opzichte van monitoring op basis van klinische beoordeling en het CD4-getal, omdat op deze wijze onnodige, dure *switches* kunnen worden voorkomen.

Hoofdstuk 15 is een literatuurstudie naar de bruikbaarheid van *dried blood spots* op filterpapier voor het meten van de viruslading en resistentiemutaties. Op basis van de in 2009 beschikbare gegevens, concludeerden we dat het meten van viruslading en resistentiemutaties van filterpapier mogelijk is, maar dat het beperkte testvolume ten koste gaat van de analytische gevoeligheid en dat opslag en transport bij hoge temperatuur en luchtvochtigheid de betrouwbaarheid van de testen negatief beïnvloedt.

Hoofdstuk 16 is een kritische beschouwing van de wijze waarop de toename van hiv-resistentie in Afrika een potentiële bedreiging vormt voor de wereldwijde aidsbestrijding. Het streven naar universele toegang tot hiv-behandeling is nog steeds de hoogste prioriteit. Daarnaast is het noodzakelijk om het functioneren van nationale hiv-behandelprogramma's te verbeteren met betrouwbare aanvoersystemen, beschikbaarheid van tests voor de viruslading en tweedelijnstherapie en degelijke surveillancesystemen voor hiv-resistentie. Deze extra investeringen nu zullen op de langere termijn lonend blijken.

TOEKOMSTPERSPECTIEF

In de eerste plaats zal internationale steun essentieel blijven om het doel te bereiken van universele toegang tot hiv-behandeling en verbetering van de kwaliteit van zorg in Afrika. PASER heeft, samen met andere initiatieven, aangetoond dat hiv-therapieresistentie in opkomst is na de grootschalige uitbreiding van behandelprogramma's. Dit nieuwe probleem kan leiden tot afname van de behandelmogelijkheden, toename van de kosten en hogere sterfte, en vormt daardoor een potentiële bedreiging voor de wereldwijde aidsbestrijding. Toekomstige hiv-behandelrichtlijnen voor ontwikkelingslanden moet rekening gaan houden met de meest recente, lokale gegevens over therapieresistentie.

Routinematige monitoring van de viruslading in het bloed verdient aanbeveling, omdat de accurate en vroegtijdige identificatie van patiënten met virologisch therapiefalen resistentieaccumulatie en onnodig switchen voorkomt, en hierdoor kostenbesparingen kan opleveren. Het verdient overweging om aangepaste eerstelijns behandeling met een bPI te introduceren in populaties met een hoge prevalentie van primaire NNRTI-resistentie, maar het is op dit moment onduidelijk bij welke grens een dergelijke

interventie kosteneffectief is. Meer studies in andere gebieden en subpopulaties zijn nodig om de evolutie van primaire resistentie in de tijd te bestuderen. Periodieke resistentiestudies, op basis van EWIs en resistentietesten, dienen te worden geïntegreerd in de routinematige kwaliteitsevaluatie van behandelprogramma's. De capaciteit voor resistentiediagnostiek in de regio moet worden uitgebreid om deze resistentiestudies mogelijk te maken. Kosteneffectiviteitsstudies zijn nuttig om te bepalen hoe we diagnostische tests en medicijnen zo optimaal mogelijk kunnen gebruiken.

CONCLUSIE

Grootschalige hiv-behandeling in Afrika heeft in het voorbije decennium miljoenen levens gered. Er zijn duidelijke aanwijzingen dat hiv-resistentie in opkomst is na de uitbreiding van hiv-behandeling. Om duurzame hiv-behandeling in ontwikkelingslanden te waarborgen, is er een dringende behoefte aan meer financiële middelen en verbetering van de infrastructuur. Internationale organisaties, nationale overheden, behandelprogramma's en donoren moeten zich gezamenlijk inspannen om het functioneren van HIV behandelprogramma's te verbeteren en daarmee op de lange termijn de effectiviteit van de huidige beschikbare behandelcombinaties te behouden.

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About the author

Raph L. Hamers (1977, The Netherlands) received his medical degree *cum laude* in 2003 from Maastricht University, The Netherlands. His enthusiasm for infectious diseases and global health ignited during internships in South Africa and Brazil. He gained a post-graduate VSBfonds study grant to work as a lecturer at the Universidade Federal de Roraima in Boa Vista, Brazil, and to conduct health services research in Brazilian primary care. After his return in The Netherlands, he worked as a resident in internal medicine (Tergooiziekenhuizen, Hilversum) and intensive care (VU Free University Medical Centre, Amsterdam). He joined PharmAccess Foundation in 2006 to establish and coordinate the *PharmAccess African Studies to Evaluate Resistance* (PASER) network in six sub-Saharan African countries, with the aim of building regional capacity for the population-based assessment of HIV drug resistance. He also co-founded the *Affordable Resistance Test for Africa* (ART-A) consortium, a public-private partnership that develops affordable test algorithms for HIV-1 drug resistance. He conducted his PhD research on drug-resistant HIV in sub-Saharan Africa at the University of Amsterdam and the Amsterdam Institute for Global Health and Development, under the supervision of Promotores Prof. dr. Tobias Rinke de Wit and Prof. dr. Peter Mugyenyi, and Co-Promotores dr. Michèle van Vugt and dr. Rob Schuurman. He presented the key study findings at the Conference on Retroviruses and Opportunistic Infections in 2011 and 2012. He serves as a WHO Expert Consultant on the HIV drug resistance assessment and prevention global strategy. He holds a post-graduate diploma in epidemiology *with distinction* from the London School of Hygiene and Tropical Medicine, University of London. Currently, he combines post-doctoral research with the internal medicine and infectious diseases residency program at the Onze Lieve Vrouwe Gasthuis and Academic Medical Center of the University of Amsterdam. Raph Hamers is married with Marlous Grijsen and they have a daughter, Lieve.