

High Rates of Transmission of Drug-resistant HIV in Aruba Resulting in Reduced Susceptibility to the WHO Recommended First-line Regimen in Nearly Half of Newly Diagnosed HIV-infected Patients

L. Marije Hofstra,^{1,2} Elena Sánchez Rivas,³ Monique Nijhuis,¹ Leonie E. A. Bank,^{1,4} Eduan Wilkinson,^{5,6} Karina Kelly,³ Tania Mudrikova,⁴ Rob Schuurman,¹ Tulio de Oliveira,^{5,6} Jaclyn de Kort,³ and Annemarie M. J. Wensing¹

¹Virology, Department of Medical Microbiology, University Medical Center Utrecht, The Netherlands; ²Department of Infection and Immunity, Luxembourg Institute of Health, Esch-sur-Alzette, Luxembourg; ³Department of Internal Medicine, Dr Horacio E. Oduber Hospital, Oranjestad, Aruba; ⁴Department of Internal Medicine and Infectious Diseases, University Medical Center Utrecht, The Netherlands; ⁵Africa Centre for Population Health, and ⁶School of Laboratory Medicine and Medical Sciences, College of Health Sciences, University of KwaZulu-Natal, Durban, Republic of South Africa

Background. In Western countries emergence of human immunodeficiency virus (HIV) drug resistance has tremendously decreased, and transmission of drug resistance has merely stabilized in recent years. However, in many endemic settings with limited resources rates of emerging and transmitted drug resistance are not regularly assessed.

Methods. We performed a survey including all HIV-infected individuals who received resistance testing in 2010–2015 in Aruba, a highly endemic HIV area in the Caribbean. Transmitted HIV drug resistance was determined using World Health Organization (WHO) criteria. Transmission dynamics were investigated using phylogenetic analyses. In a subset, baseline samples were re-analyzed using next generation sequencing (NGS).

Results. Baseline resistance testing was performed in 104 newly diagnosed untreated individuals (54% of all newly diagnosed individuals in 2010–2015): 86% were men, 39% were foreign-born, and 22% had AIDS at diagnosis. And 33% (95% CI: 24–42%) was infected with a drug-resistant HIV variant. The prevalence of resistance to non-nucleoside reverse transcriptase inhibitors (NNRTIs) reached 45% (95% CI: 27–64%) in 2015, all based on the prevalence of mutation K103N. NGS did not demonstrate additional minority K103N-variants compared to routine resistance testing. K103N-harboring strains were introduced into the therapy-unexposed population via at least 6 independent transmissions epidemiologically linked to the surrounding countries. Virological failure of the WHO-recommended first-line NNRTI-based regimen was higher in the presence of K103N.

Conclusions. The prevalence of resistant HIV in Aruba has increased to alarming levels, compromising the WHO-recommended first-line regimen. As adequate surveillance as advocated by the WHO is limited, the Caribbean region could face an unidentified rise of NNRTI-resistant HIV.

Keywords. HIV-1; drug resistance; transmission; surveillance; therapy.

Human immunodeficiency virus (HIV) infection requires combination antiretroviral therapy (cART) to prevent selection of resistance and virological failure. In Western countries, results of a genotypic baseline resistance test are taken into account when determining the first-line regimen. Patients are closely

monitored during therapy and switched timely upon virological failure. Surveys indicated that in this setting the emergence of HIV drug resistance has decreased and transmission of drug resistance (TDR) has merely stabilized in recent years [1, 2].

The HPTN-052 trial demonstrated that effective cART results in a tremendous reduction in transmission of HIV, indicating the public health potential of wide uptake of cART [3]. In recent years global efforts have increased access to cART in low- and middle-income countries [4]. Following World Health Organization (WHO) guidelines, a standard regimen consisting of 2 nucleoside reverse transcriptase inhibitors (NRTIs) and a non-NRTI (NNRTI) has been rolled out as initial cART [5]. Although this regimen has demonstrated to be very potent, its genetic barrier to resistance is low. One mutation in the viral genome is sufficient to cause high-level resistance to the NNRTI-compound. In many endemic settings with limited resources

Received 27 July 2016; editorial decision 6 January 2017; accepted 24 January 2017; published online March 9, 2017.

Correspondence: A. M. J. Wensing, University Medical Center Utrecht, G04.614, Virology, Department of Medical Microbiology, Heidelberglaan 100, 3584 CX Utrecht, The Netherlands (a.m.j.wensing@umcutrecht.nl).

Clinical Infectious Diseases® 2017;64(8):1092–97

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laboratory monitoring is limited. Although the WHO advises to perform regular surveys in these settings to monitor resistance [6], surveillance has only been implemented by a limited number of countries due to resource constraints and logistic challenges.

The Caribbean has the second highest HIV prevalence in the world (1.1%) [7]. Aruba is a constituent country of the Kingdom of the Netherlands and a relatively affluent part of the Caribbean. The densely populated island is known for its multicultural society with one-third of the population being born abroad, mainly in surrounding countries or in the Netherlands. The prevalence of HIV in Aruba is estimated at 0.5% (HIV DATA 2011-2015, Service of Contagious Diseases, unpublished) [8]. A work or residency permit will not be granted to intended immigrants who test positive for HIV. HIV treatment is free for all individuals legally registered in Aruba. Since 2010, testing for HIV drug resistance was increasingly performed. We noted a worrying rise in the detection of NNRTI-mutation K103N, which compromises the efficacy of the WHO-recommended first-line regimen. Therefore, we investigated the population infected with this resistant variant, the transmission dynamics of this virus and its impact on the outcome of cART.

METHODS

The Oduber Hospital is the only institute that provides HIV therapy in Aruba. All HIV-infected individuals who received a genotypic resistance test between 1 January 2010 and 1 of January 2016 were identified. Genotypic resistance analysis of *pol* was performed using Sanger sequencing at the UMC Utrecht and interpreted based on the IAS-USA tables [9]. Demographic, clinical, and virological data were retrieved from patient records. Ethical clearance for this study has been provided by the hospital board. Written informed consent was obtained from all participants.

TDR was determined among individuals who were tested for resistance at baseline (before exposure to therapy). Patient interviews did not reveal earlier history of antiviral treatment. The prevalence of TDR was defined as the percentage of individuals infected with a virus harboring any of the surveillance drug resistance mutations of the WHO list [10]. Baseline characteristics were compared using χ^2 , Fisher Exact, and Mann-Whitney U tests. Susceptibility to the initiated first-line regimen was assessed based on the predicted level of resistance by the Stanford HIVdb-algorithm v7.0 [11]. Viral loads were measured routinely every 3 months. Virological failure (VF) was determined as a confirmed viral load above 50 copies/mL 6 months after start of cART. A switch of cART was considered VF, except for switches of solely NRTI compounds and switches of any compound during virological suppression.

Phylogenetic Analyses

HIV-1 subtypes were determined using HIV subtyping tool COMET v0.5 [12] and REGA v3 [13]. All subtype B sequences

($n = 130$) were aligned with baseline subtype B sequences from the Netherlands ($n = 426$) and the most similar sequences selected via BLAST using MAFFT ($n = 132$) [14]. The sequences were 1257 bp long, including the full protease gene and the first 320 codons of the reverse transcriptase gene. Drug resistance related positions [10] were excluded. A maximum-likelihood (ML) tree was constructed in FastTree using the general time reversible substitution (GTR) model with gamma-distributed rate variation among sites [15]. The GTR model of evolution was estimated from the data set with ModelTest. In order to assess clade support Shimodaira-Hasegawa approximate likelihood ratio test (SH-aLRT) with 1000 pseudo-replicates was applied in FastTree. The ML tree topology was refined with 100 extra rounds of branch moves. This process was done with both nearest-neighbor interchanges and subtree-prune-regraft tree topology operators, as applied in FastTree. Transmission clusters were identified with ClusterPicker [16] from the ML tree by high branch support ($>90\%$) and intraclade genetic distance of less than or equal to 4%. In total, we identified 4 clusters associated with Aruba, the mean branch support of the 4 clusters was 99.52% (ranging from 98.6% to 99.9%), and the mean genetic diversity was 1.275% (ranging from 0.1 to 2.8%). Drug resistance mutations were annotated, and the tree was visualized in Figtree (<http://tree.bio.ed.ac.uk/software/figtree/>).

Next Generation Sequencing

A subset of baseline samples were re-analyzed using next generation sequencing (NGS). The nested polymerase chain reaction (PCR) product of the initial amplification for Sanger sequencing was used for input. Amplicons were purified using the QiaQuick PCR purification kit (Qiagen). Library preparation was done using a Nextera-XT DNA Library Preparation and Index kit (Illumina, USA) according to the manufacturer's instructions. Resulting libraries were normalized and pooled. Sequencing was performed on an Illumina MiSeq platform using the MiSeq Reagent Kit v2 for 500 cycles. To determine the background sequencing error rate, DNA plasmids of HXB2 and HXB2 with site-directed mutant K103N were included, directly and after nested PCR. Analysis was performed with DeepChek v1.4 (ABL, TherapyEdge). For each sample the Sanger sequence was uploaded in parallel. Samples with too low coverage (<2000 reads) were excluded.

RESULTS

HIV drug resistance testing was performed at baseline for 104 individuals and upon therapy failure for 28 individuals. Of all newly diagnosed HIV-infected individuals that entered into care in Aruba, the percentage receiving a resistance test at baseline increased from 26% in 2010 to 69% in 2015 (Figure 1A). The overall prevalence of TDR in this group was 33% (95% confidence interval [CI]: 24–42%). Resistance to NRTIs and PIs (both

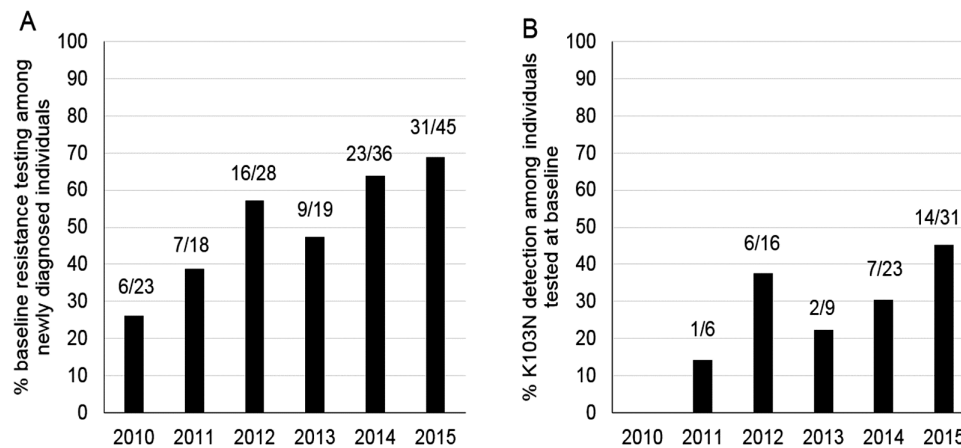


Figure 1. Percentage of newly diagnosed individuals entering into care in the Oduber Hospital in Aruba who received a resistance test at baseline (1A) and the percentage of tested individuals in whom transmission of a K103N variant was detected (1B) from 2010 until 2015.

2%; 95% CI: 0–5%) was rare, but the prevalence of NNRTI resistance was 32% (95% CI: 23–41%). All NNRTI resistance was due to mutation K103N in the reverse transcriptase encoding region. Its prevalence at baseline increased to 45% (95% CI: 27–64%) in 2015 (Figure 1B). Among individuals who were tested during therapy failure, K103N was detected in 54% (15/28).

Of the individuals tested at baseline, approximately 40% were foreign born, similar to the general population in Aruba (Table 1). The majority was male and between 30 and 50 years of age, which was in line with data on all newly diagnosed HIV patients in Aruba (HIV data 2011–2015, Service of Contagious Diseases, unpublished). Over 20% presented with an AIDS-defining illness at diagnosis. All patients were infected with an HIV-1 subtype B

virus. Patients infected with a K103N-strain were significantly more often MSM, diagnosed in more recent years and more often diagnosed during serologically confirmed recent infection compared to patients infected with wild-type virus.

Phylogenetic analysis of 688 sequences was performed to investigate the origin of the Aruban subtype B sequences and to determine the source of transmission of K103N-variants. The ML tree identified 4 transmission clusters with high-bootstrap and low sequence diversity that contained at least one individual with K103N at baseline (Figure 2). Interestingly, for 3 of the clusters we identified epidemiological links to neighboring countries. The largest cluster (1) consisted of 37 men (34 MSM), living in Aruba. They have originated from Aruba (n = 22),

Table 1. Baseline Characteristics of Individuals Who Were Tested for Drug Resistance at Baseline, Before Exposure to Antiretroviral Therapy

		Total (n = 104)	No K103N (n = 71)	K103N (n = 33)	P value
Sex	Male	89 (85.6)	58 (81.7)	31 (93.9)	.136
Age at diagnosis	Median (IQR)	41 (31–50)	43 (35–50)	37 (28–46)	.217
Year of diagnosis	Median (IQR)	2014 (2012–2015)	2013 (2011–2014)	2014 (2012–2015)	.011
Country of origin	Aruba	64 (61.5)	47 (66.2)	17 (51.5)	.318
	Colombia	15 (14.4)	10 (14.1)	5 (15.2)	
	Venezuela	5 (4.8)	2 (2.8)	3 (9.1)	
	Netherlands	5 (4.8)	1 (1.4)	4 (12.1)	
	Other	15 (14.4)	11 (15.5)	4 (12.1)	
Route of transmission	MSM	61 (58.7)	34 (47.9)	27 (81.8)	.016
	Heterosexual contact	35 (33.7)	29 (40.8)	6 (18.2)	
Country of infection	Aruba	92 (88.4)	63 (88.7)	29 (87.9)	.823
	Outside Aruba	7 (6.7)	5 (7.0)	2 (6.1)	
	Unknown	5 (4.8)	3 (4.2)	2 (6.1)	
Recent infection		14 (13.5)	5 (7.0)	9 (27.3)	.011
AIDS at diagnosis		23 (22.1)	17 (23.9)	6 (18.2)	.616
CD4 count at diagnosis	Median (IQR)	294 (76–500)	247 (64–327)	437 (218–617)	.002
Log HIV RNA load at diagnosis	Median (IQR)	5.1 (4.3–5.7)	5.1 (4.3–5.7)	5.0 (4.3–5.7)	.946
Subtype B		104 (100.0)	71 (100.0)	33 (100.0)	

Data are presented as no. (%) unless otherwise specified.

Abbreviations: HIV, human immunodeficiency virus; IQR, interquartile range; MSM, men who have sex with men.

Colombia (n = 7), Venezuela (n = 4), Cuba (n = 1), Suriname (n = 1), the Netherlands (n = 1), and India (n = 1). Mutation K103N was detected in 25/34 baseline sequences in this cluster. Three K103N cases were identified during therapy failure, of whom 2 were diagnosed with HIV in Venezuela before coming to Aruba. The basal position of the Venezuelan sequences with K103N while on therapy failure suggests that the origin of this large cluster may be associated with an external introduction of HIV-1 to Aruba. The second cluster (2) consisted of five individuals, of which 4 were from Aruba. K103N was detected in 2 individuals: the first was a basal sequence from an Aruban man who was diagnosed with HIV in Curacao after infection via MSM contact, and the second from a Dutch woman who indicated to be infected in Aruba via heterosexual contact. The remaining 2 clusters contained pairs of baseline sequences with K103N: (3) 2 Dutch MSM who both indicated to be infected in Aruba and (4) a woman who originated from and was diagnosed with HIV in the Dominican Republic and an Aruban man. Two other single baseline sequences with K103N were found, resulting in a total of 6 independent introductions of variants with K103N into the therapy naive population. The 2 single sequences were from individuals who originated from Aruba and indicated to be infected in Aruba via MSM and heterosexual contact.

The clustering of sequences with and without K103N raised the question whether reversion of K103N to wild-type had

occurred in those without K103N. NGS was successfully performed on 65/71 samples with wild-type virus and 7 control samples with K103N-virus. Based on the control plasmids, the cut-off for detecting minority variants was set at 2%. No additional minority K103N-variants were detected in any of the tested individuals compared to population sequencing. In all 7 patients with K103N at baseline, NGS confirmed the presence of this mutation at high prevalence in the viral population (varying from 94.9% to 98.8%). Three of these 7 patients have likely been infected for a long time, considering their low CD4 counts at time of diagnosis (23, 66, and 161 cells/mm³). Even in these patients we did not find evidence of reversion of K103N.

Therapy was initiated in 79/104 (76%) newly diagnosed individuals, using NNRTI-based first-line in 51 of them (65%). Regimens based on boosted protease inhibitors (PIs) or integrase inhibitors (INSTIs) were initiated in 22 (28%) and 6 (8%) individuals, respectively, mainly due to the detection of K103N at baseline. The median follow-up was 12 months. Among individuals starting a suboptimal NNRTI regimen in the presence of K103N, VF occurred in 7/9 individuals: 5 switched the NNRTI compound within 6 months and 2 did not reach virological suppression within 6 months. Among individuals with K103N who initiated fully active PI-based cART, VF was observed in one individual, with evident signs of nonadherence. In individuals with wild-type virus, VF was observed in 4/42

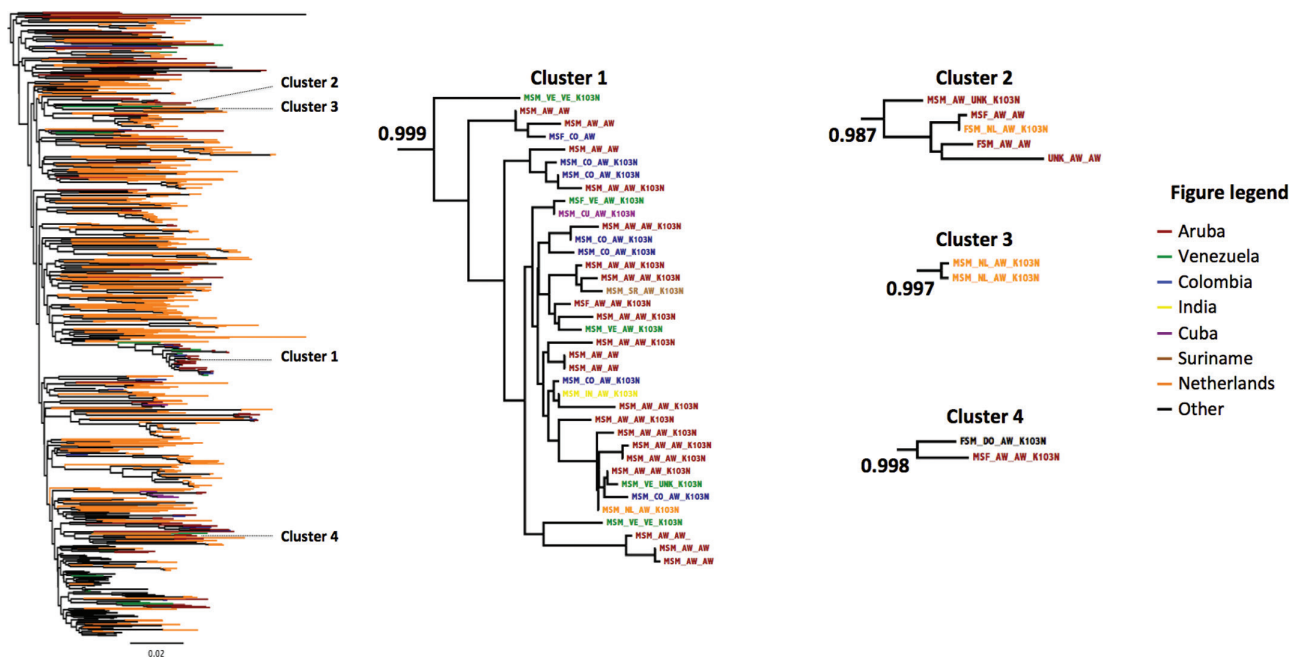


Figure 2. ML phylogenetic tree of 688 sequences, including 130 sequences from Aruba patients (marked in red), 426 baseline sequences from Dutch patients (marked in orange) and 132 sequences selected via Viroblast. Four clusters with K103N sequences are indicated and shown in more detail. Bootstrap supports are indicated at the base of the branches. The tips of the tree and sequence names are also annotated by the country of origin of the individuals (marked in different colors, according to the legend). Sequence names include reported route of transmission, the country of origin, the reported country of infection and presence of K103N. Abbreviations: AW, Aruba; CO, Colombia; CU, Cuba; DO, Dominican Republic; FSM, heterosexual woman; IN, India; MSF, heterosexual man; MSM, men who have sex with men; NL, the Netherlands; SR, Suriname; UNK, Unknown; VE, Venezuela.

(10%) individuals on an NNRTI-based regimen. Three of them selected K103N and were switched to a PI-based regimen.

DISCUSSION

This study reports an alarming rise in transmitted NNRTI resistance in therapy-naive HIV-infected individuals in an endemic country in the Caribbean. In 2015, NNRTI resistance mutation K103N was found in nearly half of the newly diagnosed individuals in Aruba who were tested for resistance before initiation of cART. This mutation causes high-level resistance to the NNRTIs efavirenz and nevirapine, which are cornerstone drugs in first-line cART regimens in high endemic areas worldwide. As such the frequent presence of this mutation at baseline is of major concern.

Aruba has a relatively small number of HIV-infected individuals, but this study raises concern on the efficacy of the WHO first-line regimen in other parts of the Caribbean and Latin America. Despite increasing coverage of cART in the Caribbean, systematic surveys to assess the selection and spread of resistant HIV are not performed. There are only scattered data available that have been recently nicely reviewed [17]. A regional analysis found no TDR in 2000–2002 when cART was only limited available [18]. Thereafter 3 surveys demonstrated an increase in NNRTI resistance to 6–7% in 2007–2011 in Cuba where cART was introduced relatively early [19–21]. Surveys in the Dominican Republic in 2007–2010 [22] and Jamaica in 2009 and 2011 [23, 24] reported 4–8% baseline NNRTI resistance. No surveys have been performed in the region since 2011. To gain more insight in the origin and spread of the resistant viruses in Aruba, we performed in depth phylogenetic analysis. We showed that transmission of K103N-variants in Aruba was frequently linked to individuals who were originating from and/or diagnosed with HIV in surrounding countries. Our data indicate that the region could face a yet unidentified rise in resistant HIV.

The increased transmission of K103N-variants could be a result of a high degree of therapy failure in individuals on efavirenz or nevirapine-based regimens. However, the overall rate of first-line regimen failure in Aruba is similar to settings in developed countries [25] where no such increase in transmission of K103N-variants has been reported [1, 2]. Phylogenetic analysis revealed that strains with K103N were introduced into the therapy-naive population multiple times, in the MSM as well as the heterosexual population. One introduction subsequently spread widely into a large cluster. The short genetic distances suggest a pattern of onward transmission by therapy-naive individuals.

It has been shown previously that particularly transmission of viruses harboring NNRTI resistance is associated with clustering [26]. Over time resistant variants may revert to wild-type after transmission to gain viral fitness [27], limiting hereby the potential for further spread of the resistant variant. However,

we have previously shown that K103N has limited impact on viral fitness *in vitro* [28] and long-term persistence of this mutation has also been observed *in vivo* [29]. In this cohort we observed persistence of mutation K103N at high frequencies despite the long duration of infection in some patients. We did not find evidence of reverted minority K103N variants with next generation sequencing in patients diagnosed with wild-type virus based on population sequencing. The long-term persistence of variants with K103N after transmission underlines their threatening potential to spread widely among therapy-naive individuals.

Regarding the consequences for clinical care, in our survey the results of resistance testing were reported to the clinicians, who often initiated an adjusted regimen if K103N was detected. This limited the possibility of statistical analyses to assess the impact on therapy outcome. Two of 4 individuals continuing a compromised NNRTI-based regimen for more than 6 months did not reach virological suppression in our cohort. This is in line with other reports showing that virological failure occurs more frequently in patients with TDR who do not receive a fully active regimen [25, 30]. In addition, rapid accumulation of resistance has been described in patients who continue a failing first-line regimen resulting in loss of future treatment options [31]. One in 5 individuals in our cohort presented with an AIDS-defining illness at diagnosis, a phenomenon also observed in neighboring countries [32, 33]. Particularly in these patients immediate intervention with fully active cART is crucial to prevent further disease progression and mortality.

A limitation of our study is that not all newly diagnosed patients received baseline resistance testing. Retrospective testing was not possible due to the unavailability of stored plasma. The incomplete testing was a result of gradual uptake of baseline resistance testing and not based on a specific selection. Our random sample included more than half of all newly diagnosed patients in 2010–2015 and their baseline characteristics were similar to those reported in health monitoring reports of all newly diagnosed patients in this period (HIV data 2011–2015, Service of Contagious Diseases, unpublished). As such, we expect that the introduced bias was limited.

Emergence of HIV drug resistance during cART use has tremendously decreased in Western countries, and transmission of drug resistance has merely stabilized in recent years [1, 2], as a result of close laboratory monitoring, the use of regimens with a higher genetic barrier to resistance, and surveillance. However, our data demonstrate that in a setting without adequate surveillance, the prevalence of infections with NNRTI resistant HIV can increase to worrisome levels, compromising the efficacy of the standard first-line NNRTI-based regimen. Following our findings, local and regional public health authorities have been informed. Local guidelines have reinforced baseline resistance

testing and replaced the WHO recommended first-line regimen by an integrase inhibitor-based regimen. Our data indicate that it is crucial to combine the scale up of cART in low- and middle-income countries with adequate surveillance for resistance as recommended by the WHO [6], to guard the efficacy of our current public health approach and to prevent jeopardizing the potential benefit of treatment as prevention. If strengthened surveillance would show that these NNRTI resistant viruses are more widespread in the region, a change of the current first-line NNRTI-based regimen to a regimen with a higher genetic barrier to resistance should be strongly considered, despite its major implications for resources and logistics.

Notes

Acknowledgments. We thank Wouter Nijhuis and Petra van Ham for their contribution to next generation sequencing. We also want to acknowledge Luis Chong for his supportive role in retrieving the data.

Funding. This work was supported in part by the Fonds National de la Recherche Luxembourg (FNR) [CORE C12/BM/4011111] and an unrestricted educational grant provided by Gilead. E. W. and T. d. O. are funded by a Medical Research Council flagship grant of the Republic of South Africa (MRC-REA-UFSP-01-2013/UKZN HIVEPI). The funders had no role in study design, data collection, analysis, or interpretation, or preparation of the manuscript.

Potential conflicts of interest. A. M. J. W. has received an unrestricted educational grant from Gilead for the conduct of the study and reports grants, travel support or consultancy fees from Viiv Healthcare, Gilead, CLJI, Janssen, BMS, MSD and Virology Education outside of the submitted work. M. N. reports grants from Shanghai de Novo Pharmtech and GSK outside of the submitted work. Other authors have no conflicts to declare. All authors have submitted the ICMJE Form for Disclosure of Potential Conflicts of Interest. Conflicts that the editors consider relevant to the content of the manuscript have been disclosed.

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