Limited clinical benefit of minority K103N and Y181C-variant detection in addition to routine genotypic resistance testing in antiretroviral therapy-naive patients

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Objective: The presence of minority nonnucleoside reverse transcriptase inhibitor (NNRTI)-resistant HIV-1 variants prior to antiretroviral therapy (ART) has been linked to virologic failure in treatment-naive patients.

Design: We performed a large retrospective study to determine the number of treatment failures that could have been prevented by implementing minority drug-resistant HIV-1 variant analyses in ART-naïve patients in whom no NNRTI resistance mutations were detected by routine resistance testing.

Methods: Of 1608 patients in the Swiss HIV Cohort Study, who have initiated first-line ART with two nucleoside reverse transcriptase inhibitors (NRTIs) and one NNRTI before July 2008, 519 patients were eligible by means of HIV-1 subtype, viral load and sample availability. Key NNRTI drug resistance mutations K103N and Y181C were measured by allele-specific PCR in 208 of 519 randomly chosen patients.

Results: Minority K103N and Y181C drug resistance mutations were detected in five out of 190 (2.6%) and 10 out of 201 (5%) patients, respectively. Focusing on 183 patients for whom virologic success or failure could be examined, virologic failure occurred in seven out of 183 (3.8%) patients; minority K103N and/or Y181C variants were present prior to ART initiation in only two of those patients. The NNRTI-containing, first-line ART was effective in 10 patients with preexisting minority NNRTI-resistant HIV-1 variant.

Conclusion: As revealed in settings of case–control studies, minority NNRTI-resistant HIV-1 variants can have an impact on ART. However, the implementation of minority NNRTI-resistant HIV-1 variant analysis in addition to genotypic resistance testing (GRT) cannot be recommended in routine clinical settings. Additional associated risk factors need to be discovered. © 2014 Wolters Kluwer Health | Lippincott Williams & Wilkins

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Introduction

HIV type 1 (HIV-1) establishes a chronic infection that is incurable to date. Combination antiretroviral therapy (ART) substantially decreases the morbidity and mortality in HIV-1 infected patients [1]. To achieve this outcome, the majority of patients have to be treated lifelong. This goes along with the risk of virologic failure and development of drug resistance that limits further treatment options [2,3]. Furthermore, as drug-resistant viruses can be transmitted, it is recommended to test for the presence of drug resistance mutations before initiating ART in treatment-naive patients [4].

Routine genotypic resistance testing based on population sequencing (GRT) misses minority drug-resistant HIV-1 variants representing less than 20–25% of the virus population [5–7]. The application of more sensitive technologies to detect and quantify minority drug-resistant HIV-1 variants has revealed that these low abundant viruses can cause virologic failure especially in the context of ART regimens with a low genetic barrier to resistance, for instance, nonnucleoside reverse transcriptase inhibitor (NNRTI)-containing regimens [8–11]. A pooled analysis of 10 studies and almost 1000 patients has shown that preexisting minority NNRTI-resistant HIV-1 variants are associated with virologic failure [9]. A recent European multicohort case–control study [11] of 368 individuals using centralized ultrasensitive 454 sequencing showed a significantly increased risk of virologic failure to first-line NNRTI-based ART when minority drug-resistant HIV-1 variants preexisted. These studies were designed as case–control or cohort studies focusing on patients experiencing virologic failure [9].

Current antiretroviral regimens are very efficient and the number of virologic failures due to drug resistance is low in ART-naive patients. However, to minimize this risk of virologic failure of first-line ART even further, it is currently discussed to implement more sensitive genotypic resistance testing in routine clinical settings to detect systematically preexisting minority drug-resistant HIV-1 variants. To answer this, we have designed a study composed of a representative group of patients from the nation-wide, observational Swiss HIV Cohort Study (SHCS). Patients selected for inclusion were ART-naive, started with a NNRTI-containing ART and in whom no NNRTI or NRTI resistance mutation was detected by routine GRT prior to ART. Minority drug-resistant HIV-1 variants were measured and analysed in the context of virologic success or failure.

Materials and methods

Study participants and study design

The SHCS is an observational, nation-wide cohort, including at least 45% of the cumulative number of HIV-1 infected individuals declared to the Swiss health authorities [12]. The inclusion criteria for this substudy were as follows: chronic infection with HIV-1 subtype B or 01_AE, start of first-line ART with NNRTI and two NRTIs before 1 July 2008, no NNRTI or NRTI resistance mutations prior to ART detected by population sequencing, baseline viral load above 1000 HIV-1 RNA copies/ml plasma and available plasma sample within 6 months before initiation of first-line ART.

Random selection was performed within strata of HIV-1 subtype and time periods (4-year intervals of estimated year of HIV-1 infection between 1980 and 2008; time band sampling). The number of samples per stratum was selected proportionally to the fraction observed in all eligible patients. The infection date was estimated according to the method developed by P. Taffé and M. May [13]. The SHCS has been approved by ethical committees from all participating institutions and written informed consent has been obtained from all individuals [12].

Twenty-four months follow-up data were used to differentiate two groups of patients: those who experienced virologic failure and those who were successfully treated. Virologic failure was defined as virologic rebound with two consecutive viral loads more than 200 HIV-1 RNA copies/ml plasma after having achieved a viral load less than 50 copies/ml after initiation of first-line ART or one viral load of more than 200 HIV-1 RNA copies/ml plasma after 6–24 months of continuous treatment, followed by a modification of treatment or as virologic nonresponse, that is failing to achieve a decrease in viral load less than 200 HIV-1 RNA copies/ml plasma within 16 weeks of continuous treatment.

Viral load, resistance testing and allele-specific real-time PCR

Plasma HIV-1 viral load was quantified and genotypic resistance testing by population sequencing was

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performed as described [14]. Drug resistance mutations were defined as recommended by the International Antiviral Society-USA (IAS-USA) Drug Resistance Mutations Group and the surveillance drug resistance mutations list [2,15]. Minority K103N, Y181C and M184V drug-resistant HIV-1 variants were quantified by allele-specific real-time PCRs (AS-PCRs) as previously described in detail [10,14,16–18]. Each AS-PCR has a dynamic range of six logs and a detection limit of 10 HIV-1 DNA copies per reaction. The discriminatory abilities are as follows: 0.01% for the K103N, 0.2% for the Y181C and M184V mutations. Mutational load was calculated by multiplying the percentage of a given minority drug-resistant HIV-1 variant with the viral load.

Data and statistical analyses

Clinical data from the SHCS were used as well as HIV-1 *pol* sequences from the SHCS drug resistance database, which are stored in SmartGene's Integrated Database Network System (IDNS version 3.6.6) [19]. Statistical analyses were performed with Stata 12 SE software (Stata Corp., College Station, Texas, USA). All *P* values were two-sided, and the level of significance was set at 0.05.

Results

Baseline characteristics

The SHCS comprised 15 857 participants on 30 June 2010. One thousand, six hundred and eight participants were chronically HIV-1 infected, ART-naive and started first-line ART containing one NNRTI and two NRTIs before 1 July 2008 (Fig. 1). Another inclusion criterion was the infection with HIV-1 subtype B or 01_AE, which was necessary, because the AS-PCR primer design is only optimal for those two subtypes. For other HIV-1 subtypes, individual confirmation of the homology of primer binding sites would have been necessary that could potentially have jeopardized the random selection of patients. As shown in Fig. 1, 519 patients were deemed eligible for this study, of whom 221 individuals were randomly selected for minority drug-resistant HIV-1 variant testing. Finally, data from 208 patients were included in the analysis. Thirteen exclusions were due to sample unavailability, previously not reported NNRTI or NRTI resistance in the majority of viruses, or unsuccessful RT-PCR (Fig. 1).

These 208 patients were representative of all 519 eligible patients, thus representing chronically HIV-1 subtype B or 01_AE infected patients in Switzerland receiving

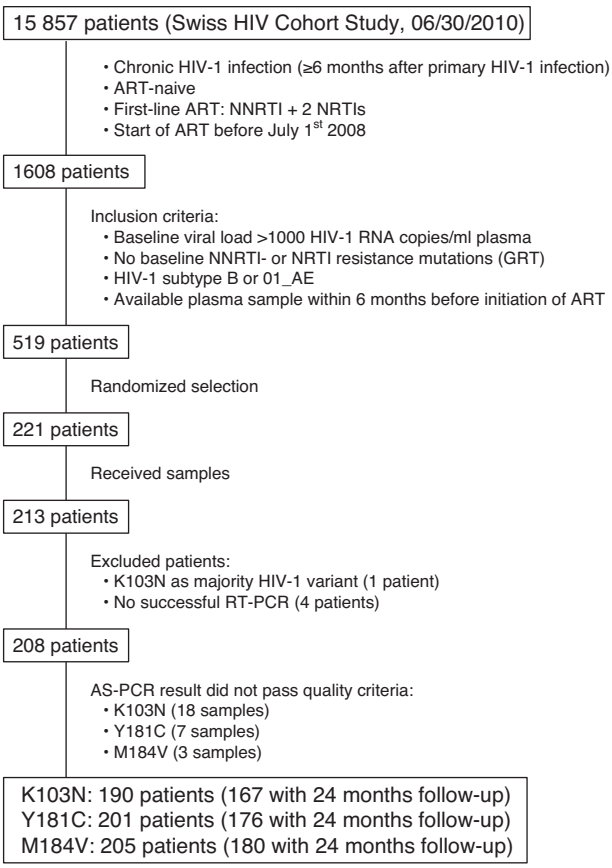

Fig. 1. Scheme of study design.

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first-line, NNRTI-containing ART. No significant differences were observed in terms of age, sex, ethnicity, route of transmission, estimated year of HIV-1 infection, $CD4^+$ cell count and viral load at baseline, and first-line ART regimens (Table 1). It has to be noted that certain clinical parameters differed significantly between the 519 eligible patients and the remaining 1089 of all 1608 patients who started first-line treatment with an NNRTI-containing regimen. Due to the exclusion of almost all HIV-1 non-B subtypes, these groups differed in terms of sex distribution, transmission routes, ethnicity and, naturally, HIV-1 subtype distribution (data not shown). Patients who matched our inclusion criteria were predominantly white MSM and were infected with HIV-1 subtype B. Both groups did not differ in terms of the rate of virologic failure, viral load prior to initiation of ART and first-line NNRTI.

Low prevalence of preexisting minority nonnucleoside reverse transcriptase inhibitor-resistant HIV-1 variants in antiretroviral therapy-naive patients in routine clinical settings and missing association with virologic failure

Minority NNRTI-resistant HIV-1 variants were present prior to first-line NNRTI-containing ART in 14 of 208 patients (6.7%). The K103N mutation was detected in five out of 190 (2.6%) patients at frequencies of 0.08–7.46% (3 x <1%, 2 x >2%) and the Y181C mutation in 10 out of 201 (5%) patients at frequencies of 0.5–2.7% (7 x <1%, 2 x 1–2%, 1 x >2%). Calculating the mutational load, that is the total amount of HIV-1 RNA copies/ml plasma carrying a drug resistance mutation, the results were as follows: K103N: 104–20 579 (3 x <1000, 2 x >2000) and Y181C: 215–6489 (9 x <1000, 1 x >2000) mutant HIV-1 RNA copies/ml plasma. The M184V mutation was verified in one of 205 (0.5%) patients representing 0.9% of the virus population, that is 91 mutant HIV-1 RNA copies/ml plasma. Virus replication was successfully suppressed in this patient. Figure 2 depicts 12/14 patients who were followed-up for 24 months.

For 183 of the 208 randomly chosen patients, virologic success or failure could be examined, because 24 or more months of follow-up was available. Twenty-five patients were not eligible for this analysis due to the following reasons: two patients died, two were lost to follow-up, 13 patients switched to another regimen due to side effects, four patients decided to switch or stop treatment, and in four patients the reason for discontinuation of treatment was unknown; virological failure was not reported, and two patients harboured the Y181C mutation as minority variant.

Of those 183 patients, 176 patients (96.2%) were virologically suppressed until month 24 while receiving their first-line, NNRTI-containing regimen. Seven patients (3.8%) experienced virologic failure as defined

Table 1. Baseline characteristics of randomized/tested patients and remaining patients of a total of 519 eligible patients in the investigation of usefulness of implementing more sensitive drug resistance assays in routine clinical settings.

Clinical parameter		Randomized/tested patients ($n=208$)	Not tested patients ($n=311$)	P
Age, median (IQR), years		39 (32–45)	39 (34–45)	0.159
Sex, No. (%)	Male	170 (81.7)	263 (84.6)	0.395
	Female	38 (18.3)	48 (15.4)	
Ethnicity, no. (%)	White	188 (90.4)	279 (89.7)	0.802
	Nonwhite	20 (9.6)	32 (10.3)	
Route of transmission, no. (%)	MSM	114 (54.8)	178 (57.2)	0.841
	Het	67 (32.2)	93 (29.9)	
	IDU	27 (13.0)	40 (12.9)	
HIV-1 subtype, no. (%)	B	198 (95.2)	292 (93.9)	0.527
	01_AE	10 (4.8)	19 (6.1)	
Estimated year of HIV-1 infection, median (IQR), years		1999 (1995–2001)	1998 (1995–2001)	0.582
$CD4^+$ cell count median (IQR), cells/μl blood		218.5 (159.5–292)	216 (150–308)	1
HIV-1 viral load, median (IQR), log10 HIV-1 RNA copies/ml plasma		4.9 (4.5–5.3)	4.9 (4.4–5.2)	0.276
start of first-line ART, median (IQR), years		2005 (2003–2007)	2005 (2002–2006)	0.086
NNRTI, no. (%)	EFV	180 (86.5)	275 (88.4)	0.522
	NVP	28 (13.5)	36 (11.6)	
NRTI, no. (%)	ZDV and 3TC	73 (35.1)	135 (43.4)	0.295
	TDF and FTC	61 (29.3)	86 (27.6)	
	TDF and 3TC	31 (14.9)	44 (14.2)	
	ABC and 3TC	21 (10.1)	21 (6.8)	
	Others	22 (10.6)	25 (8.0)	
Virologic outcome of first-line ART, no. (%)		$n=183$	$n=288$	
	Virologic success	176 (96.2)	269 (93.4)	0.199
	Virologic failure	7 (3.8)	19 (6.6)	

ABC, abacavir; 3TC, lamivudine; EFV, efavirenz; FTC, emtricitabine; Het, heterosexual; HIV-1, human immunodeficiency virus type 1; IDU, intravenous drug use; IQR, interquartile range; MSM, men who have sex with men; NNRTI, nonnucleos/tide reverse transcriptase inhibitor; NRTI, nucleos/tide reverse transcriptase inhibitor; NVP, nevirapine; TDF, tenofovir; ZDV, zidovudine.

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in the Materials and Methods section (Table 1). Two of them harboured minority NNRTI-resistant HIV-1 variants prior to first-line ART (Fig. 2). In patient 8, who failed with the exclusive selection of the M184I mutation, 0.6% of the virus population carried the Y181C mutation prior to ART. Patient 13, who harboured both NNRTI mutations in high mutational loads prior to ART, failed rapidly within the first 4 months, accompanied by the selection of K103N and Y181C mutations (Fig. 2). For this patient, a likely association existed between preexisting minority NNRTI-resistant HIV-1 variants and virologic failure. However, the virologic failures in the other five patients cannot be explained by the preexistence of minority

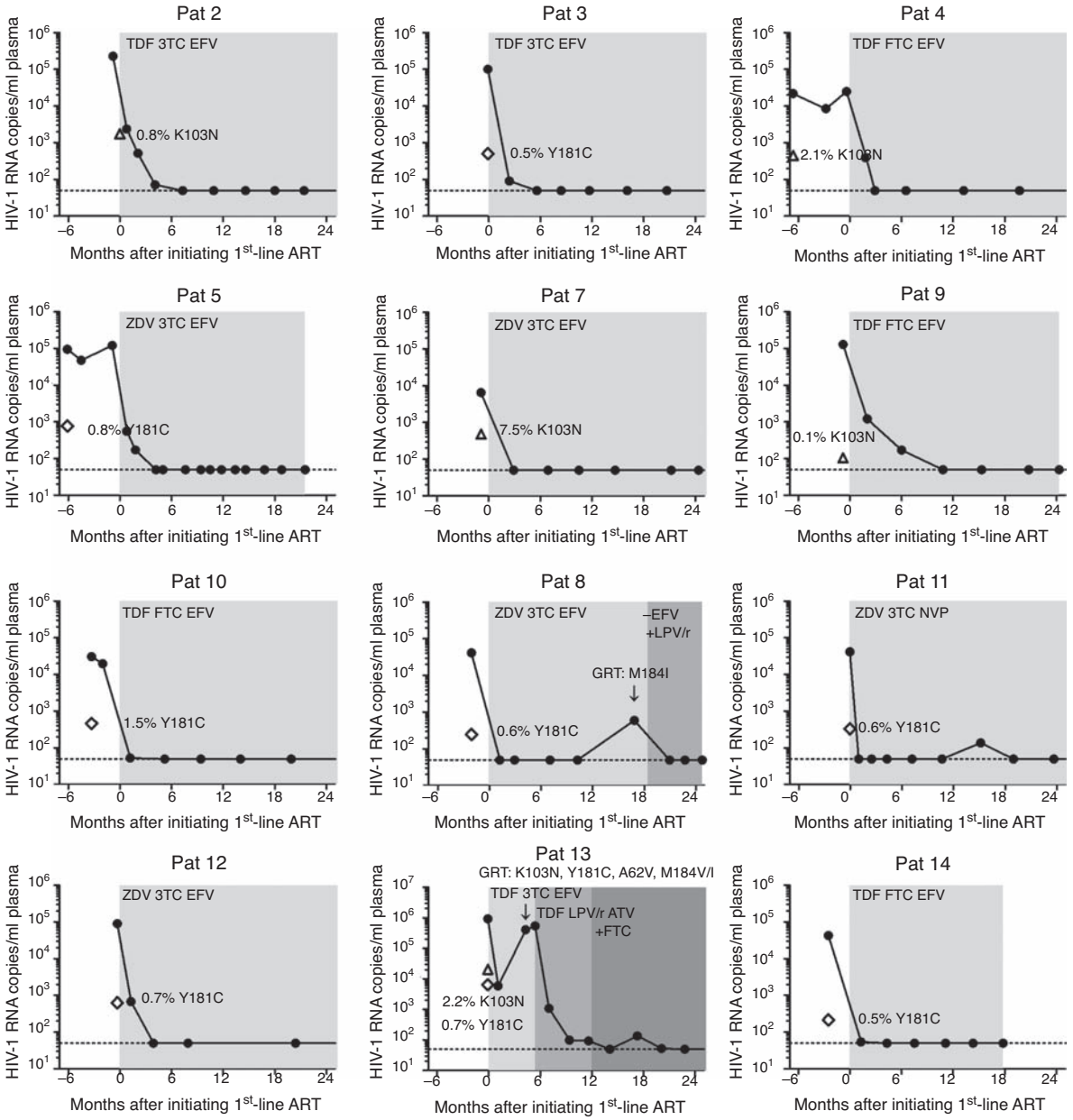

Fig. 2. Preexisting minority nonnucleoside reverse transcriptase inhibitor-resistant HIV-1 variants and virologic outcome of first-line, nonnucleoside reverse transcriptase inhibitor containing antiretroviral therapy. Plasma viral load was measured using Cobas AmpliPrep/Cobas TaqMan HIV-1 Test, versions 1 and 2.0 (black circles). The limit of detection was fixed to 50 HIV-1 RNA copies/ml plasma and is shown in dashed lines. Minority K103N (open triangles) and Y181C (open diamonds) HIV-1 variants were quantified by AS-PCR; percentages are given and were used to calculate the absolute amounts of these variants based on the viral load. Periods of ART are depicted in grey shaded areas. /r, ritonavir-boosted; 3TC, lamivudine; ART, antiretroviral therapy; ATV, atazanavir; EFV, efavirenz; FTC, emtricitabine; GRT, genotypic resistance testing; LPV, lopinavir; NVP, nevirapine; TDF, tenofovir; ZDV, zidovudine.

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K103N or Y181C HIV-1 variants. In contrast, another 10 patients harboured minority NNRTI-resistant HIV-1 variants prior to first-line ART without experiencing virologic failure (Fig. 2).

As the number of well defined virologic failures was low (seven patients of whom just two harboured preexisting minority NNRTI-resistant HIV-1 variants), we measured minority NNRTI-resistant HIV-1 variants in additional 10 patients from the 19 not tested patients experiencing virologic failure (Table 1). The K103N mutation was not detected in any of those patients. The Y181C mutation was detected in one of 10 patients prior to ART at a frequency of 0.5%, that is 725 Y181C RNA copies/ml plasma. This patient showed the following drug resistance mutations during virologic failure: K65R, K103N, Y181C and M184V. In total, three of 13 patients carrying minority NNRTI-resistant HIV-1 variants prior to first-line ART experienced virological failure. This results in a positive predictive value for any of these minority NNRTI resistance mutations of 23% meaning that first-line, NNRTI-containing ART would be wrongfully withheld from a large proportion of patients. Vice versa, in a total of 17 patients experiencing virological failure, only three harboured minority NNRTI-resistant variants prior to ART.

Discussion

We performed an investigation to test the hypothesis that additional implementation of more sensitive assays for the detection of minority drug-resistant HIV-1 variants in routine clinical settings would prevent those few virologic failures in treatment-naive patients who are initiating ART with a NNRTI-containing regimen, although the routine genotypic resistance test based on population sequencing does not detect any NNRTI resistance mutation. The SHCS reported low virologic failure rates in patients receiving an NNRTI-containing, first-line regimen: 45 out of 805 (6%) patients starting ART between 1999 and 2005 [19]. Our most recent analysis, including all patients between 1999 and June 2008, showed no change in the rate of virologic failure (84/1309; 6.4%). And our inclusion criteria, such as HIV-1 subtype B or 01_AE, did not change this either (26/471; 5.5%). Minority NNRTI-resistant HIV-1 variants were measured by AS-PCR in 208 randomly chosen patients to determine whether more sensitive genotypic resistance testing would have prevented those virologic failures. Similar to the low rate of virologic failure, the prevalence of preexisting minority NNRTI-resistant HIV-1 variants was low. Yet, the observed overlap was minimal, meaning that only two out of seven patients with virologic failure harboured preexisting minority NNRTI-resistant HIV-1 variants, whereas only two out of 12 patients with preexisting minority NNRTI-resistant HIV-1 variants experienced virologic failure. Minority M184V variants were irrelevant in our cohort.

These results are contradictory to a pooled analysis of 10 studies [9] and a recent European multicohort case–control study, both showing an increased risk of virologic failure to first-line NNRTI-based ART when minority drug-resistant HIV-1 variants were detected prior to ART [11]. However, when separately examining each of these studies included in the pooled analysis [9], it shows that all four case–control studies resulted in a significant association between virologic failure and preexisting minority drug-resistant HIV-1 variants in ART-naive individuals [10,20–22], but four of the included six cohort studies did not reveal this association [23–26]. In terms of the other two cohort studies, an association was found when the mutational load was more than 2000 K103N copies/ml plasma [27] or when all preexisting NNRTI-resistance mutations were included in the analysis independent of their frequency [28]. In the meantime, three other cohort studies have been published and none of them show an association between virologic failure and preexisting minority drug-resistant HIV-1 variants in ART-naive individuals [29–31]. This failure in detecting an association could be due to the small number of individuals included in most of the cohort studies ($n < 100$ in each study), but in the most recently published study, more than 200 individuals were included [29].

By measuring only the K103N, Y181C and M184V mutations, we might have missed other important and maybe more predictive drug resistance mutations. However, almost all analyses on the impact of preexisting minority drug-resistant HIV-1 variants on NNRTI-containing regimens are based on AS-PCR focusing exactly on these three mutations, which are key drug resistance mutations [9]. It might be possible to close this gap by applying next-generation technologies, but this technology has currently a lower sensitivity than AS-PCR [32,33]. Noteworthy, expanding the spectrum of measured drug resistance mutations would not change the observation that most of the patients harbouring preexisting minority NNRTI-resistant HIV-1 variants were successfully treated with an NNRTI-containing regimen. Thus, for those patients, NNRTIs would have been wrongly withheld.

The mutational load, that is the absolute number of viruses harbouring drug resistance mutations within the viral population, might be more predictive for virologic failure than the percentage of minority drug-resistant variants. It has been shown that a preexisting mutational load of more than 2000 K103N HIV-1 RNA copies/ml plasma, quantified by AS-PCR specific for this mutation, is highly predictive of virologic failure in patients starting with a first-line, NNRTI-containing regimen [27].

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Patient 13 harboured more than 20 000 K103N and more than 6000 Y181C HIV-1 RNA copies/ml plasma prior to ART, whereas the mutational load was less than 2000 copies/ml in all other patients. Also, in a recent pooled analysis of almost 1000 patients, an increase of the mutational load was associated with an increased risk of virologic failure, but the risk increased already significantly at low mutational loads of 10–99 copies/ml plasma [9].

We studied the impact of preexisting minority NNRTI-resistant HIV-1 variants on the outcome of NNRTI-containing, first-line ART in a retrospective manner. Undoubtedly, a prospective study would be the ultimate concept to proof or to deny the relevance of those minority NNRTI-resistant HIV-1 variants on the outcome of first-line regimens, particularly for treatments with a low genetic barrier to resistance. But due to the low rate of virologic failure, the low prevalence of preexisting minority NNRTI-resistant HIV-1 variants in ART-naive patients and a variety of other factors such as the numerous possibilities of ART regimens, a huge number of patients would need to be included in such a study.

Cost effectiveness was not explicitly analysed in our study. Our data show that applying a more sensitive drug resistance assay in 100 patients would have prevented one virologic failure. However, in the case of preexisting minority NNRTI-resistant HIV-1 variants, potentially prevented failures have to be balanced against the costs of NNRTI-sparing regimens, which often are more costly and less often available as single tablet regimens. As virologic failure did not occur in most of the patients with preexisting minority NNRTI-resistant HIV-1 variants, this suggests that standard pretreatment screening for minority variants will not be the most cost-effective option.

In summary, our study shows that preexisting K103N, Y181C or M184V variants only have a very low positive predictive value for ART-naive patients in routine clinical settings wherein the rate of virologic failure is very low as it is the case for the SHCS [34]. Hence, the number of possibly preventable virologic failures in patients harbouring minority NNRTI-resistant HIV-1 variants was small. Noteworthy, most virologic failures occurred without evidence for preexisting minority NNRTI-resistant HIV-1 variants, and in contrast, most of the patients harbouring minority NNRTI-resistant variants did not experience virologic failure. Although per-existing minority NNRTI-resistant HIV-1 variants may have an impact on ART as revealed in settings of case–control studies, the implementation of minority NNRTI-resistant HIV-1 variant analysis in addition to GRT cannot be recommended in routine clinical settings. Additional associated cofactors need to be discovered.

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Conflicts of interest

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