MAJOR ARTICLE

Efficient Suppression of Minority Drug-Resistant HIV Type 1 (HIV-1) Variants Present at Primary HIV-1 Infection by Ritonavir-Boosted Protease Inhibitor–Containing Antiretroviral Therapy

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Background. Selection of preexisting minority variants of drug-resistant human immunodeficiency virus type 1 (HIV-1) can lead to virological failure in patients who receive antiretroviral therapy (ART) with low genetic resistance barriers. We studied treatment response and dynamics of minority variants during the first weeks of ART containing a ritonavir–boosted protease inhibitor (PI) and 2 nucleoside reverse-transcriptase inhibitors (NRTIs), which is a regimen with a high genetic resistance barrier.

Methods. Plasma samples obtained prior to initiation of ART from 109 patients with primary HIV infection and samples obtained during viral decay during early ART from 17 of these 109 patients were tested by allele-specific polymerase chain reaction for K103N and M184V variants.

Results. K103N and/or M184V mutations were detected in 15 (13.8%) of 109 patients prior to ART as minority variants. No selection of these variants was observed within the first weeks of ART in 7 of 15 patients with preexisting drug resistance mutations, nor was any selection observed in 10 patients without preexisting drug resistance mutations. Most patients received ART immediately after diagnosis of HIV-1 infection, showed a rapid decrease in viral load, and experienced sufficient suppression of viremia for $\leqslant$48 months.

Conclusions. Minority variants, in particular viruses harboring the M184V mutation, were efficiently suppressed in patients with acute infection who received a ritonavir-boosted PI and 2 NRTIs (most regimens included lamivudine). Under this high genetic resistance barrier regimen, the M184V was not further selected.

The reduction of morbidity and mortality among patients with human immunodeficiency virus type 1 (HIV-1) infection is one of the major achievements in the treatment of infectious diseases [1]. Moreover, the approval of new drugs within the already broad range of reverse-transcriptase inhibitors and protease inhibitors (PIs) and new drug classes are constantly increasing treatment options [2]. However, eradication of HIV-1 is not feasible with current regimens [3–5]; thus, development of drug-resistant viruses is still a major concern. Virological failure limits further treatment

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options because of cross-resistance and persistence of drug-resistant viruses [6, 7].

Because of possible transmission of drug-resistant viruses [8–12], it is recommended to perform resistance testing before initiation of antiretroviral therapy (ART) [13], because preexisting drug-resistant viruses can lead to therapy failure when they represent the majority of viral variants [14, 15]. Resistance testing is usually performed using population sequencing, which does not allow detection of drug-resistant virus populations representing less than 20%–25% of the variants [16, 17]. Allele-specific real-time PCR (AS-PCR) can bridge this gap by allowing the detection of minority variants with discriminatory abilities as low as 0.01% [18].

We and others have shown that minority drug-resistant HIV-1 variants can be detected despite the absence of drug resistance mutations by population sequencing. This was clearly demonstrated in women after treatment with single-dose nevirapine to prevent mother-to-child transmission [19, 20], in acute seroconverters [21, 22], and in patients undergoing structured treatment interruptions [23]. Furthermore, it was shown that preexisting minority drug-resistant variants can lead to virological failure in treatment-experienced patients [24–27]. However, the impact of these drug-resistant viruses at low frequencies in treatment-naive patients initiating ART is still controversial. Several studies did not observe a correlation between their presence prior to ART and virological failure, and other studies reported a correlation, especially in the context of ART regimens with a low genetic resistance barrier [22, 28–35]. These diverse observations might be caused by differences with regard to the methodology used and their discriminatory abilities, patient populations (eg, patients with acute or recent infection vs those with long-term infection), follow-up periods, and antiretroviral regimens chosen for first-line treatment displaying dissimilarities in genetic resistance barriers.

Here, we investigated a possible correlation between the clinical outcome of ART regimens with a high genetic resistance barrier containing a ritonavir–boosted PI, together with 2 nucleoside reverse-transcriptase inhibitors (NRTIs) and the presence of minority drug-resistant HIV-1 variants in a group of 109 patients with acute HIV-1 infection from the Zurich primary HIV-1 infection study (ZPHI) prior to ART by AS-PCR for the K103N and M184V variants. Furthermore, possible early selection of drug-resistant viruses was measured by AS-PCR for the K103N and M184V variants in longitudinal plasma samples of the first weeks of ART of 17 patients harboring and patients not harboring drug-resistant viruses at low frequencies prior to ART.

METHODS

Study design and patients. Plasma samples from 109 patients from the ZPHI cohort were obtained from March 2002 through December 2007 from edetic acid blood samples collected 0–19 days before initiation of ART. For 17 of 109 patients, 1–4 plasma samples per patient were collected during viral decay within the first 16 weeks of ART. The ZPHI study is an observational, open label, nonrandomized, single-center study (ClinicalTrials .gov identification no. NCT00537966). Patients with acute and recent HIV-1 infection are included. Acute HIV-1 infection is defined as (1) presentation of the acute retroviral syndrome (ARS) and negative or indeterminate Western-blot test results in the presence of a positive p24 antigen test result and/or detectable viral load or (2) documented seroconversion with or without symptoms within 90 days. Recent infection is defined as (1) possible ARS, a positive Western-blot test result, detectable viral load, and a negative HIV gp120 avidity respectively detuned assay result [36] or (2) documented acute HIV-1 infection with referral to our center >90 days after presumed date of infection. Patients are offered early ART and, after 1 year of suppressed viremia (<40 HIV-1 RNA copies/mL of plasma), patients can chose to interrupt ART. Written informed consent was obtained from each patient prior to inclusion. Clinical follow-up data for the patients until December 2008 have been analyzed. Thus, all patients had ⩾12 months of follow-up.

HIV quantification and resistance testing. Plasma HIV-1 RNA was quantified using the Cobas AmpliPrep/Cobas TaqMan HIV-1 test (Roche Diagnostics) with a limit of 40 HIV-1 RNA copies/mL of plasma. Resistance testing by population sequencing was performed using the Viroseq vs 1 (PE Biosystems) or Viroseq vs 2 (Abbott). Drug resistance mutations were defined as recommended by the International AIDS Society–USA Drug Resistance Mutations Group and the surveillance drug resistance mutations list [13, 37].

AS-PCR for detection and quantification of minority drug-resistant HIV-1 variants harboring K103N and/or M184V mutations. Viral RNA from 1 mL of plasma was analyzed retrospectively by AS-PCR quantifying minority variants of viruses harboring the K103N or the M184V mutation. Processing of plasma, reverse transcription, first amplification, the K103N and M184V AS-PCR assays, and the data analysis have been described elsewhere [21, 23]. The K103N AS-PCR has been further validated in a blinded, multicenter comparison of methods detecting minority variants [38]. The discriminatory abilities of the K103N and M184V AS-PCR assays are 0.01% and 0.2% for the respective mutant. Because the discriminatory ability is not only dependent on the limitations of each AS-PCR assay but also on the viral load, individual cutoff values were calculated on the basis of viral load, as described elsewhere [23].

Reverse-transcriptase sequences were individually checked on the basis of data obtained by population sequencing with regard to primer binding sites for all patients infected with

HIV-1 subtype non-B. Patients were not included in this study when >3 mutations were observed within 1 primer binding site of the primers used for the first amplification or of the primer used for the reverse transcription. In addition, total copy numbers obtained by AS-PCR had to be in due proportion to the corresponding viral load from the same time point. Outliers have been excluded from final analyses. All samples were repeated when 1 or both mutations were detected at low frequencies. Furthermore, each run, in addition to standard dilutions and no template master mix samples, contained 4 controls: (1) 10^7 DNA copies of wild-type and mutant standard separately amplified with the respective incorrect allele-specific primer to control the discriminatory ability of each AS-PCR run; (2) 10.000 HIV-1 NL4–3 wild-type RNA copies in 1 mL plasma of an HIV-1 negative donor (wild-type control); (3) mixture of 9.900 HIV-1 NL4–3 wild-type RNA copies plus 100 HIV-1 NL4–$3_{K103N+M184V}$ RNA copies in 1 mL of plasma of an HIV-1–negative donor (1% mutant control); and (4) 1 mL of plasma of an HIV-1–negative donor (negative control). Each time, 13 plasma samples from patients and the controls were processed in parallel, starting with RNA isolation, and measured in the same K103N and M184V AS-PCR runs. Performance of DNA standard dilutions and controls of each AS-PCR were compared with previously performed AS-PCR assays. Thus far, >100 AS-PCR runs regarding each of the mutations K103N and M184V have been performed. The complete run was repeated when, for example, 1 standard was out of range.

Statistical analysis. The impact of the presence of minority drug-resistant HIV-1 variants at baseline was statistically assessed using the 2-tailed Wilcoxon rank-sum test for continuous data and the 2-tailed Fisher's exact test or $\chi 2$ test for categorical data. Statistical analyses were performed using the software Prism5 (GraphPad Software). *P* values <.05 were regarded as statistically significant.

RESULTS

Drug resistance mutations at low frequencies in primary HIV-1 infection prior to ART. All patients are asked to participate in the ZPHI study who are seen in our outpatient clinic and private practices in Zurich, Switzerland, who show clinical signs and have diagnostic values that indicate acute or recent infection. From January 2002 through December 2007, 164 patients were consecutively included in the ZPHI study, of whom 50 patients were not eligible for the retrospective analysis of drug resistance mutations at low frequencies because of the following reasons: the HIV-1 genotype revealed too many mismatches in the primer binding sites for the AS-PCR assays (27 patients with HIV-1 subtype non-B), no plasma sample was available that had been obtained prior to ART initiation (15 patients), the patient dropped out of the study (5 patients), or the presence of chronic HIV-1 infection was detected at the time of entry into the study (3 patients). Thus, AS-PCR assays were performed using plasma samples from 114 patients. Samples from 5 patients were repeatedly classified as outliers because of no amplification or amplification that was too weak. The number of mismatches between HIV-1 genotype and oligonucleotides could not explain the outliers (4 patients were infected with HIV-1 subtype B). No obvious reason for these outliers could be determined; however, these 5 patients were excluded from the final analyses.

The presence of minority variants of K103N-harboring and M184V-harboring viruses was measured by AS-PCR in plasma samples from 109 patients with primary HIV-1 infection prior to ART initiation. Neither of these 2 mutations were detected by resistance testing using population sequencing in any of these patients. In 4 patients (3.7%), major drug resistance mutations were detected by population sequencing (M41L and L210W [reverse transcriptase]; L90M [protease]; M46L [protease]; and D67N [reverse transcriptase]). Treatment failure was not observed in these patients.

AS-PCR was applied to the earliest available plasma samples with regard to diagnosis of HIV-1 infection. In the majority of patients who initiated ART, the plasma sample was obtained on the same day that early ART was initiated. The K103N mutation was detected in 6 (5.5%) of 109 patients at frequencies of 0.08%–3.76% (Table 1). In 11 (10.1%) of 109 patients, the M184V mutation was found as minority variants representing 0.4%–8.3% of plasma viral genomes (Table 1). In 2 patients, both mutations were detected. In total, viruses obtained from 15 (13.8%) of 109 patients harbored the K103N and/or the M184V mutations at low frequencies. All patients were offered early ART containing a ritonavir-boosted PI and 2 NRTIs as standard regimen at their first visit; thus, the decision was not based on the HIV-1 genotype. Only 2 patients received initial therapy with 1 nonnucleoside reverse-transcriptase inhibitor (NNRTI) and 2 NRTIs, because they wished to receive a once-daily regimen. Both patients switched to a ritonavir-boosted PI plus 2 NRTIs within the first weeks of ART because of adverse effects. When patients declined to initiate early ART, they could still participate in the ZPHI cohort as untreated control patients.

Comparison of patients harboring and patients not harboring minority drug-resistant HIV-1. Both groups were compared with regard to sex, age, route of transmission, presence of ARS, acute and recent infection, $CD4^+$ cell count and HIV-1 RNA copy numbers at baseline, HIV-1 subtype, and drug resistance, as detected by population sequencing. None of these parameters were significantly different between the groups (Table 2). The majority of patients were men who have sex with men and were infected with HIV-1 subtype B. ARS was diagnosed in >85% of patients, and acute HIV-1 infection was

Table 1. Detection of Minority Variants of K103N and M184V Viruses in 15 of 109 Patients with Primary Human Immunodeficiency Virus Type 1 (HIV-1) Infection Prior to Antiretroviral Therapy

Patient no.	Sex	Route of infection	HIV-1 subtype	Year of primary HIV-1 infection	HIV-1 RNA level, copies/mL of plasma	Allele-specific real-time PCR		Drug resistance mutations by GRT	First- line treatment
						K103N, mean % (±SD)	M184V, mean % (±SD)		
28023	M	MSM	B	2002	11,000	<LLD	2.4 ± 1.1	None	LPV/r, AZT, 3TC
108	M	MSM	B	2003	3560	<LLD	1.0 ± 0.1	None	LPV/r, AZT, 3TC
109	M	MSM	B	2003	55,200	<LLD	1.3 ± 0.1	None	LPV/r, AZT, 3TC
115	M	MSM	B	2003	6040	0.91 ± 0.24	1.0 ± 0.0	None	LPV/r, AZT, 3TC
121	M	MSM	B	2003	1550	<LLD	2.1 ± 0.2	None	LPV/r, AZT, 3TC
123	M	MSM	B	2003	3,620,000	0.18 ± 0.02	<LLD	None	LPV/r, AZT, 3TC
131	M	MSM	B	2004	3,335,000	<LLD	0.4 ± 0.0	None	LPV/r, TDF, ddI
135	M	MSM	B	2004	226,000	0.83 ± 0.30	8.3 ± 2.2	None	None
137	M	MSM	B	2004	87,300	<LLD	0.4 ± 0.0	None	LPV/r, AZT, 3TC
162	M	MSM	B	2005	51,100	<LLD	0.4 ± 0.0	None	LPV/r, AZT, 3TC
172	M	MSM	B	2005	47,900	1.25 ± 0.13	<LLD	None	LPV/r, AZT, 3TC
177	M	MSM	B	2005	2,220,000	3.76 ± 0.79	<LLD	None	LPV/r, AZT, 3TC
179	F	Heterosexual sex	B	2005	60,900	<LLD	2.1 ± 1.0	None	None
194	M	MSM	B	2006	930,000	<LLD	0.4 ± 0.1	None	LPV/r, AZT, 3TC
214	M	MSM	06_CPX	2006	52,800	0.08 ± 0.03	<LLD	None	LPV/r, AZT, 3TC

NOTE. 3TC, lamivudine; AZT, zidovudine; ddI, didanosine; GRT, genotypic resistance testing by population sequencing; LLD, lower limit of detection; LPV/r, ritonavir-boosted lopinavir; MSM, men who have sex with men; PCR, polymerase chain reaction; TDF, tenofovir.

confirmed in 78.9% of all patients for the time at which the baseline plasma sample was collected.

No selection of viruses carrying the K103N or M184V mutation during viral decay within the first 16 weeks of ART. Thirteen of 15 patients who harbored minority drug-resistant HIV-1 variants started ART during primary HIV-1 infection, and 2 patients did not receive early ART. Possible selection of these drug-resistant variants has been investigated by measuring the K103N and M184V mutations by AS-PCR in longitudinal plasma samples obtained from 7 of 13 patients who received early ART within the first weeks after treatment initiation. Because plasma samples were not suitable for AS-PCR when the viral load was <300 HIV-1 RNA copies/mL, the remaining 6 patients could not be included because of very rapid decreases in viral load. In addition, 10 patients without evidence of drug-resistant viruses at low frequencies at baseline were included in this study. They were matched to the other 7 patients with regard to viral load and $CD4^+$ T cell count at baseline, duration until viral load became undetectable, and time of infection. All 17 patients were male, were infected with HIV-1 subtype B, and received ritonavir-boosted lopinavir plus zidovudine plus lamivudine, except for 1 patient who was treated with ritonavir-boosted lopinavir plus tenofovir plus didanosine. Two patients had minority K103N-harboring HIV-1 variants before initiation of ART, which had not been selected during the first weeks. In patient 177, this variant was efficiently suppressed. At baseline, >3% of viruses carried the K103N mutation, and ~4 weeks later, this mutation was undetectable, with a detection limit of 1.4% based on the viral load at this time point (Figure 1). The M184V mutation was also not selected in any of the 5 patients who harbored this mutation at low frequencies prior to ART; however, it must be considered that, at all time points after initiation of ART, the individually estimated discriminatory thresholds of the M184V AS-PCR assay were higher than the frequencies at which the M184V was detected at baseline. For instance, patient 137 harbored 0.4% M184V viral variants prior to ART. This variant was not detected at the 2 subsequent time points with individual cutoff values based on the viral loads of 0.9% and 2.8%. This means that M184V variants could have been selected up to these individual cutoff values. However, suppression of viral replication was sufficiently obtained for all patients, which argues against rapid selection of drug-resistant viruses. No evidence for the appearance and selection of K103N or M184V variants was observed in the 10 patients who lacked these mutations prior to ART (Figure 1).

Long-term follow-up of all patients. The outcome of ART in both groups of patients was investigated. Eighty-two of 94 patients who did not harbor and 13 of 15 patients who harbored minority drug-resistant HIV-1 variants at baseline started early ART (Table 3). Among treated patients, follow-up clinical data for ⩾12 months was available for all 13 of the patients who did harbor and 79 of the 82 patients who did not harbor minority drug-resistant HIV-1 variants at baseline. All patients received a ritonavir-boosted PI and 2 NRTIs, except 1 patient who started ART containing efavirenz plus tenofovir plus emtricitabine, however, the regimen was changed at week 2.5 by replacing efavirenz with ritonavir-boosted lopinavir. All patients were offered the opportunity to interrupt ART after 12 months of efficient treatment (ie, viral load <40 HIV-1 RNA copies/mL of plasma for ⩾12 months). No significant differences were

Table 2. Characteristics of Patients with Primary Human Immunodeficiency Virus Type 1 (HIV-1) Infection Classified with Regard to the Presence of Minority Drug-Resistant HIV-1 Variants Prior to Antiretroviral Therapy

Clinical parameter	Patients haboring minority drug-resistant HIV-1 variants ($n=15$)	Patients not haboring minority drug-resistant HIV-1 variants ($n=94$)	P[a]
Sex			
Male	14 (93.3)	88 (93.6)	>.99
Female	1 (6.7)	6 (6.4)	
Age, median years (IQR)	38 (33–42)	35 (30–41)	.29
Route of transmission			
MSM	14 (93.3)	76 (80.9)	.82
Heterosexual sex	1 (6.7)	14 (14.9)	
IDU	0 (0.0)	1 (1.1)	
MSM or heterosexual sex	0 (0.0)	2 (2.1)	
IDU or heterosexual sex	0 (0.0)	1 (1.1)	
Acute retroviral syndrome			
Yes	12 (80.0)	82 (87.2)	.21
No	3 (20.0)	7 (7.4)	
ND	0 (0.0)	5 (5.3)	
Acute or recent infection			
Acute	13 (86.7)	73 (77.7)	.53
Recent	2 (13.3)	14 (14.9)	
ND	0 (0.0)	7 (7.4)	
$CD4^+$ cell count, median cells/mm^3 blood (IQR)	488 (346–553)	363 (281–568)	.48
HIV-1 RNA level, median log_{10} copies/mL plasma	4.7 (4.0–6.0)	5.3 (4.7–6.2)	.10
HIV-1 subtype			
B	14 (93.3)	85 (90.4)	>.99
Non B	1 (6.7)	9 (9.6)	
Resistance detected by population sequencing			
No	15 (100.0)	90 (95.7)	>.99
Yes	0 (0.0)	4 (4.3)	

NOTE. Data are no. (%) of patients, unless otherwise indicated. IDU, injection drug use; IQR, interquartile range; MSM, men who have sex with men; ND, not determined.

[a] Statistical analyses were performed using the 2-tailed Wilcoxon rank-sum test for continuous data and the 2-tailed Fisher's exact test or χ^2 test for categorical data.

observed in the percentages of patients interrupting ART or in the duration of ART until treatment interruption in both groups (Table 3). Furthermore, the follow-up period for patients who continued ART was not significantly different between patients who harbored and those who did not harbor K103N and/or M814V variants at low frequencies. One patient experienced treatment failure, defined by an increase in viral load >40 copies/mL of plasma after achieving <40 copies/mL at ⩾2 consecutive visits (Table 3). Viremia rapidly decreased within the first 4 months of ART in this patient; however, in the following 16 months, viral load fluctuated between 47 and 166 HIV-1 RNA copies/mL of plasma. Viral load was undetectable only once. This patient lacked drug-resistant viruses according to population sequencing and K103N and M184V AS-PCR assays that were performed before treatment initiation. None of the patients who harbored minority drug-resistant HIV-1 variants prior to ART experienced therapy failure.

DISCUSSION

The ZPHI is a long-term, observational, open label, nonrandomized, single-center study that was started in 2002. Until the end of 2007, >150 patients with acute or recent HIV-1 infection have been included. We retrospectively measured the presence of minority drug-resistant HIV-1 variants harboring K103N and/or M184V mutations using AS-PCR in the earliest available plasma samples prior to early ART for 109 individuals. None of these mutations were detected by population sequencing in samples obtained from any patient. One or both mutations were detected at low frequencies in 13.8% of patients, and the M184V mutation was more frequently observed than was the

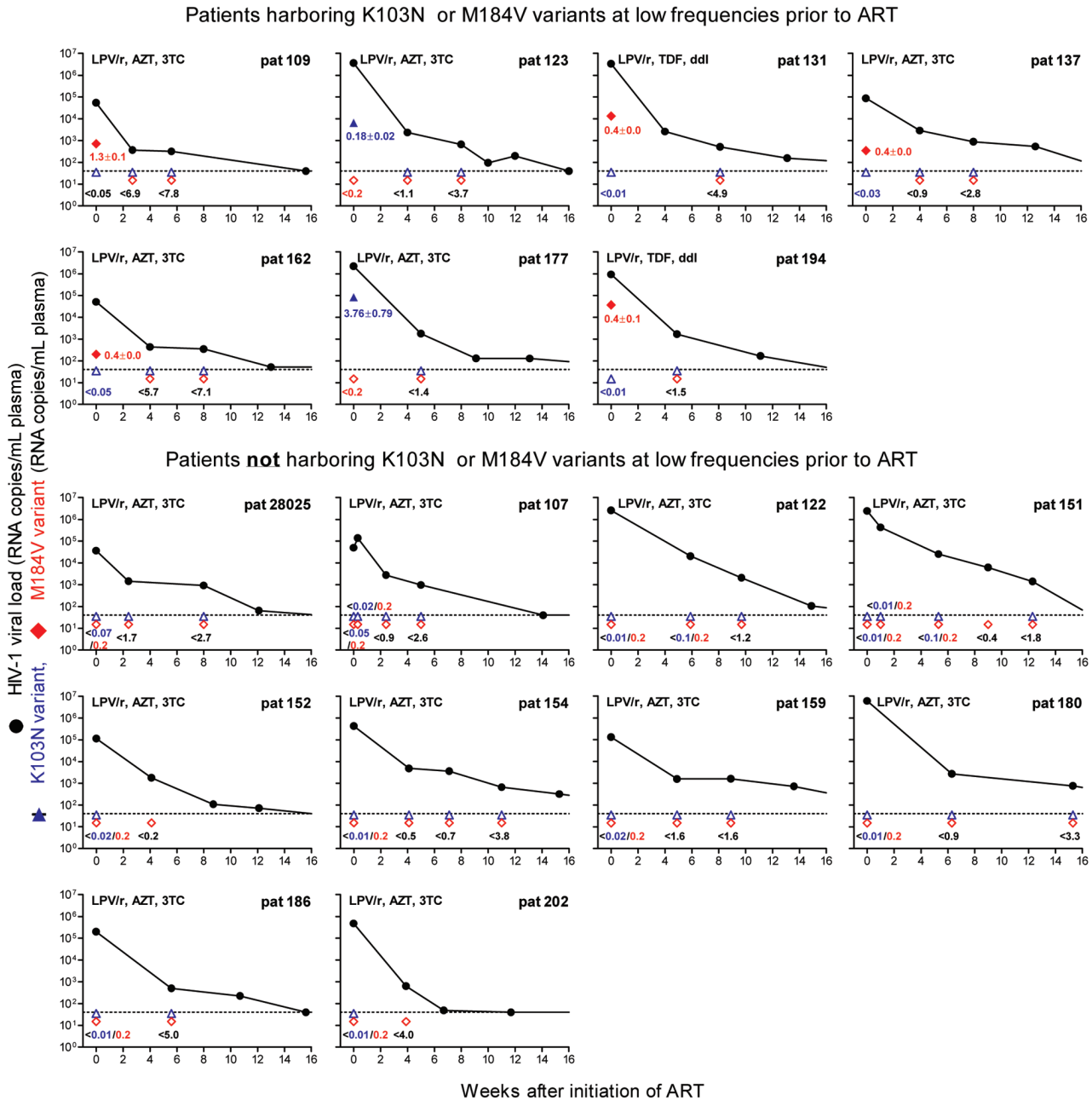

Figure 1. Kinetics of viral load and quantification of minority drug-resistant human immunodeficiency virus type 1 (HIV-1) variants in patients harboring and patients not harboring drug-resistant viruses at low frequencies prior to antiretroviral therapy (ART) during the first 16 weeks of ART. HIV RNA in plasma was measured using the Cobas AmpliPrep/Cobas TaqMan HIV-1 Test *(black circles)* with a limit of 40 copies/mL of plasma *(dashed line)*. Allele-specific polymerase chain reaction was used to quantify minority variants of K103N *(blue triangles)* and M184V *(red diamonds)* harboring viruses. The percentage of the virus population carrying the specific mutation was used to calculate the absolute HIV RNA copies/mL of plasma of the drug-resistant variants on the basis of the corresponding viral load measurement. Percentages (± standard deviations) of each drug-resistant virus population are given. With regard to each measured plasma sample without detection of K103N *(blue open triangles)* and M184V *(red open diamonds)* variants at low frequencies, discriminatory limits are shown, which have been individually calculated on the basis of the viral load. 3TC, lamivudine; AZT, zidovudine; ddI, didanosine; LPV/r, ritonavir-boosted lopinavir; TDF, tenofovir.

K103N mutation. This pattern is in accordance with low-frequency prevalence data for primary HIV-1 infection from Germany [21], France [22], and Canada [39].

We have studied the fate of those drug-resistant viruses that are present at low frequencies during the first weeks of ART. Minority drug-resistant HIV-1 variants were efficiently suppressed in all patients who harbored these variants prior to ART and included in this longitudinal analysis. Furthermore,

Table 3. Antiretroviral Therapy (ART) Regimens, Duration of ART, and Treatment Outcome for Patients with Primary Human Immunodeficiency Virus Type 1 (HIV-1) Infection Classified with Regard to the Presence of Minority Drug-Resistant HIV-1 Variants Prior to ART

Variable	Patients haboring minority drug-resistant HIV-1 variants (n = 15)	Patients not haboring minority drug-resistant HIV-1 variants (n = 94)	P[a]
Initial ART regimen			
2 NRTIs plus RTV-boosted PI	13 (86.7)	80 (85.1)	.85
2 NRTIs plus NNRTI	0 (0.0)	2 (2.1)	
No ART	2 (13.3)	12 (12.8)	
No. of patients receiving ART and followed-up for at least 12 months after initiation of ART	13	79	
ART failure			
Yes	0/13 (0.0)	1/79 (1.3)	>.99
No	13/13 (100.0)	78/79 (98.7)	
Treatment interruption			
No	6/13 (46.2)	43/79 (54.4)	.77
Yes	7/13 (53.8)	36/79 (45.6)	
Duration of ART until treatment interruption, median months (IQR)	14.2 (11.9–17.5)	16.3 (14.1–20.9)	.19
Duration of ART for patients without treatment interruption, median months (IQR)	23.3 (18.6–40.7)	17.4 (13.3–29.0)	.20

NOTE. Data are no. (%) of patients, unless otherwise indicated. IQR, interquartile range; NRTI, nucleoside reverse transcriptase inhibitor; PI, protease inhibitor.

[a] Statistical analyses were performed using the 2-tailed Wilcoxon rank-sum test for continuous data and the 2-tailed Fisher's exact test or χ^2 test for categorical data.

neither K103N-harboring nor M184V-harboring viruses were selected in these 10 patients without evidence for drug-resistant viruses before treatment initiation. All patients showed rapid decreases in viral load. The fact that no selection of the M184V variant occurred in any individual to levels above the cutoff values based on viral loads, which was, on average, 2.6%, suggests that residual replication is very low or perhaps even completely inhibited, because the M184V mutation could otherwise be rapidly and frequently selected (ie, obtain a majority) when inhibition of viral replication is insufficient. This correlation was clearly shown for patients who received dual therapy containing lamivudine and ziduvudine [40–42], patients who received triple NRTIs [43, 44], and patients who experienced early therapy failure with 2 NRTIs and 1 NNRTI [33]. Furthermore, we have previously shown that selection of drug-resistant viruses within the first weeks of ART, as determined by AS-PCR, was associated with slow decreases in viremia, which again was associated with insufficient suppression of viral replication [28]. Thus, potency of ART regimens is one important factor with regard to the avoidance of early selection of drug-resistant viruses.

However, it is probably not potency alone that can prevent the selection of preexisting viruses at low frequencies. We have recently reported that patients can experience early virological failure caused by rapid selection of preexisting minority drug-resistant HIV-1 variants despite the receipt of potent regimens [33]. Others have also shown that receipt of potent regimens with low genetic barriers to resistance is associated with virological failure when drug-resistant viruses are present at low frequencies prior to ART [30, 34]. Such correlations have not been observed in patients who received regimens that included a ritonavir-boosted PI [34] (ie, regimens with a higher genetic barrier to resistance) [45, 46]. Almost all patients in our study initiated an ART regimen that contained a ritonavir-boosted PI. The K103N mutation should have had no impact at all on such patients. Our data suggest that the M184V mutation as minor population does not influence the outcome of ART within the first 12 months in patients who receive a ritonavir-boosted PI. Ritonavir-boosted PIs have been successfully used in monotherapy trials, although treatment with ritonavir-boosted PIs is inferior to treatment with combination ART [47]. This suggests that drug resistance mutations within the reverse transcriptase might not have a major impact on the clinical outcome of ART when a ritonavir-boosted PI is part of the regimen.

It has recently been shown that having ⩾2000 RNA copies/mL of minority variants harboring the K103N mutation, rather than the percentage of minority variants harboring the K103N mutation, is associated with virological failure of NNRTI-containing regimens [48]. In our study, only 3 patients harbored ⩾2000 M184V RNA copies prior to ART. One of these patients did not start early ART, and another patient was treated with

ritonavir-boosted lopinavir, tenofovir, and didanosine (a regimen not affected by the M184V mutation). The remaining patient was early treated with ritonavir-boosted lopinavir, zidovudine, and lamivudine and did not experience virological failure. Nevertheless, it is still unknown whether such a limit exists in terms of the M184V mutation in the context of ART containing a ritonavir-boosted PI.

Prediction of virological failure of first-line ART is still not feasible based on the detection of minority drug-resistant HIV-1 variants because of controversial data, some of which shows and some of which does not show an association between the presence of drug-resistant viruses at low frequencies and virological failure [22, 28–35]. These diverse observations might be caused by differences in the discriminatory abilities of different technologies used for detection of minority variants, differences in patient populations (those with acute or recent infection vs those with long-term infection), differences in time periods of follow-up, and differences in antiretroviral regimens chosen for first-line treatment displaying dissimilarities in genetic resistance barriers and potencies. Furthermore, all studies have been retrospectively analyzed and included only limited numbers of patients who experienced virological failure. Before more-sensitive assays for detection of drug-resistant viruses at low frequencies can be implemented into routine diagnostic procedures clinical cutoff values must be defined. Admittedly, this is a complex issue in which ART potency, genetic barriers to resistance, weighting factors for individual drug resistance mutations and their linkages, and the quantity of those minority variants (in percentages or as absolute HIV RNA copy numbers), may have to be taken into consideration.

In summary, we have shown that viruses that harbor K103N and/or M184V mutations can be detected at low frequencies in a substantial number of patients with primary HIV-1 infection lacking detection of drug-resistant viruses by population sequencing. However, these drug-resistant variants were efficiently suppressed by early ART regimens that contained a ritonavir-boosted PI and 2 NRTIs. Furthermore, virological failure did not occur in these patients, indicating that ART regimens with a high genetic barrier to resistance did not select for these drug-resistant variants from 12 through 48 months. Our data support the hypothesis that the genetic barrier to resistance is an important factor with regard to the question of whether minority drug-resistant HIV-1 variants are associated with virological failure. Additional studies are necessary to define the significance of drug-resistant viruses at low frequencies before more-sensitive assays to detect drug-resistant viruses might be implemented into routine diagnostic procedures.

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References

1. Egger M, Hirschel B, Francioli P, et al. Impact of new antiretroviral combination therapies in HIV infected patients in Switzerland: prospective multicentre study. Swiss HIV Cohort Study. BMJ **1997**; 315: 1194–1199.
2. Flexner C. HIV drug development: the next 25 years. Nat Rev Drug Discov **2007**; 6:959–966.
3. Finzi D, Hermankova M, Pierson T, et al. Identification of a reservoir for HIV-1 in patients on highly active antiretroviral therapy. Science **1997**; 278:1295–1300.
4. Wong JK, Hezareh M, Gunthard HF, et al. Recovery of replication-competent HIV despite prolonged suppression of plasma viremia. Science **1997**; 278:1291–1295.
5. Chun TW, Nickle DC, Justement JS, et al. Persistence of HIV in gut-associated lymphoid tissue despite long-term antiretroviral therapy. J Infect Dis **2008**; 197:714–720.
6. Daar ES, Richman DD. Confronting the emergence of drug-resistant HIV type 1: impact of antiretroviral therapy on individual and population resistance. AIDS Res Hum Retroviruses **2005**; 21:343–357.
7. Metzner KJ. Persistence of drug-resistant HIV-1 and possible implications for antiretroviral therapy. Future Virology **2006**; 1:377–391.
8. Yerly S, Kaiser L, Race E, Bru JP, Clavel F, Perrin L. Transmission of antiretroviral-drug-resistant HIV-1 variants. Lancet **1999**; 354:729–733.
9. Grant RM, Hecht FM, Warmerdam M, et al. Time trends in primary HIV-1 drug resistance among recently infected persons. JAMA **2002**; 288:181–188.
10. Little SJ, Holte S, Routy JP, et al. Antiretroviral-drug resistance among patients recently infected with HIV. N Engl J Med **2002**; 347:385–394.
11. Masquelier B, Bhaskaran K, Pillay D, et al. Prevalence of transmitted HIV-1 drug resistance and the role of resistance algorithms: data from seroconverters in the CASCADE collaboration from 1987 to 2003. J Acquir Immune Defic Syndr **2005**; 40:505–511.
12. Yerly S, von Wyl V, Ledergerber B, et al. Transmission of HIV-1 drug resistance in Switzerland: a 10-year molecular epidemiology survey. AIDS **2007**; 21:2223–2229.
13. Hirsch MS, Gunthard HF, Schapiro JM, et al. Antiretroviral drug resistance testing in adult HIV-1 infection: 2008 recommendations of an International AIDS Society-USA panel. Clin Infect Dis **2008**; 47:266–285.
14. Durant J, Clevenbergh P, Halfon P, et al. Drug-resistance genotyping in HIV-1 therapy: the VIRADAPT randomised controlled trial. Lancet **1999**; 353:2195–2199.
15. Baxter JD, Mayers DL, Wentworth DN, et al. A randomized study of antiretroviral management based on plasma genotypic antiretroviral resistance testing in patients failing therapy. CPCRA 046 Study Team for the Terry Beirn Community Programs for Clinical Research on AIDS. AIDS **2000**; 14:F83-F93.
16. Gunthard HF, Wong JK, Ignacio CC, Havlir DV, Richman DD. Comparative performance of high-density oligonucleotide sequencing and dideoxynucleotide sequencing of HIV type 1 pol from clinical samples. AIDS Res Hum Retroviruses **1998**; 14:869–876.
17. Schuurman R, Demeter L, Reichelderfer P, Tijnagel J, de Groot T, Boucher C. Worldwide evaluation of DNA sequencing approaches for identification of drug resistance mutations in the human immunodeficiency virus type 1 reverse transcriptase. J Clin Microbiol **1999**; 37: 2291–2296.

18. Metzner KJ. Detection and significance of minority quasispecies of drug-resistant HIV-1. J HIV Ther **2006**; 11:74–81.
19. Flys T, Nissley DV, Claasen CW, et al. Sensitive drug-resistance assays reveal long-term persistence of HIV-1 variants with the K103N nevirapine (NVP) resistance mutation in some women and infants after the administration of single-dose NVP: HIVNET 012. J Infect Dis **2005**; 192:24–29.
20. Johnson JA, Li JF, Morris L, et al. Emergence of drug-resistant HIV-1 after intrapartum administration of single-dose nevirapine is substantially underestimated. J Infect Dis **2005**; 192:16–23.
21. Metzner KJ, Rauch P, Walter H, et al. Detection of minor populations of drug-resistant HIV-1 in acute seroconverters. AIDS **2005**; 19:1819–1825.
22. Peuchant O, Thiebaut R, Capdepont S, et al. Transmission of HIV-1 minority-resistant variants and response to first-line antiretroviral therapy. AIDS **2008**; 22:1417–1423.
23. Metzner KJ, Bonhoeffer S, Fischer M, et al. Emergence of minor populations of human immunodeficiency virus type 1 carrying the M184V and L90M mutations in subjects undergoing structured treatment interruptions. J Infect Dis **2003**; 188:1433–1443.
24. Kapoor A, Jones M, Shafer RW, Rhee SY, Kazanjian P, Delwart EL. Sequencing-based detection of low-frequency human immunodeficiency virus type 1 drug-resistant mutants by an RNA/DNA heteroduplex generator-tracking assay. J Virol **2004**; 78:7112–7123.
25. Lecossier D, Shulman NS, Morand-Joubert L, et al. Detection of minority populations of HIV-1 expressing the K103N resistance mutation in patients failing nevirapine. J Acquir Immune Defic Syndr **2005**; 38: 37–42.
26. Roquebert B, Malet I, Wirden M, et al. Role of HIV-1 minority populations on resistance mutational pattern evolution and susceptibility to protease inhibitors. AIDS **2006**; 20:287–289.
27. Svarovskaia ES, Margot NA, Bae AS, et al. Low-level K65R mutation in HIV-1 reverse transcriptase of treatment-experienced patients exposed to abacavir or didanosine. J Acquir Immune Defic Syndr **2007**;46: 174–180.
28. Metzner KJ, Allers K, Rauch P, Harrer T. Rapid selection of drug-resistant HIV-1 during the first months of suppressive ART in treatment-naive patients. AIDS **2007**; 21:703–711.
29. Metzner KJ, Walter H, Rauch P, et al. The prevalence of drug-resistant virus as a minority quasispecies before initiating antiretroviral therapy (ART) is not associated with therapy failure in persons initiating therapy with truvada plus PI/r or NNRTI [abstract 879]. In: Program and abstracts of the 15th Conference on Retroviruses and Opportunistic Infections, Boston, Massachusetts, **2008**.
30. Johnson JA, Li JF, Wei X, et al. Minority HIV-1 drug resistance mutations are present in antiretroviral treatment-naive populations and associate with reduced treatment efficacy. PLoS Med **2008**; 5:e158.
31. Paredes R, Lalama CM, Ribaudo HJ, et al. Pre-existing minority drug-resistant HIV-1 variants, adherence, and risk of antiretroviral treatment failure. J Infect Dis **2010**; 201:662–671.
32. Stekler J, Ellis G, Carlsson J, et al. Prevalence of low-level mutations in primary HIV-1 infection and its effect on ART [abstract 674]. In: Program and abstracts of the 16th CROI, Montreal, Canada, **2009**.
33. Metzner KJ, Giulieri SG, Knoepfel SA, et al. Minority quasispecies of drug-resistant HIV-1 that lead to early therapy failure in treatment-naive and -adherent patients. Clin Infect Dis **2009**; 48:239–247.
34. Simen BB, Simons JF, Hullsiek KH, et al. Low-abundance drug-resistant viral variants in chronically HIV-infected, antiretroviral treatment-naive patients significantly impact treatment outcomes. J Infect Dis **2009**; 199:693–701.
35. Balduin M, Oette M, Daumer MP, Hoffmann D, Pfister HJ, Kaiser R. Prevalence of minor variants of HIV strains at reverse transcriptase position 103 in therapy-naive patients and their impact on the virological failure. J Clin Virol **2009**; 45:34–38.
36. Rusert P, Kuster H, Joos B, et al. Virus isolates during acute and chronic human immunodeficiency virus type 1 infection show distinct patterns of sensitivity to entry inhibitors. J Virol **2005**; 79:8454–8469.
37. Bennett DE, Camacho RJ, Otelea D, et al. Drug resistance mutations for surveillance of transmitted HIV-1 drug-resistance: 2009 update. PLoS ONE **2009**; 4:e4724.
38. Halvas EK, Aldrovandi GM, Balfe P, et al. Blinded, multicenter comparison of methods to detect a drug-resistant mutant of human immunodeficiency virus type 1 at low frequency. J Clin Microbiol **2006**;44: 2612–2614.
39. Toni TA, Asahchop EL, Moisi D, et al. Detection of human immunodeficiency virus (HIV) type 1 M184V and K103N minority variants in patients with primary HIV infection. Antimicrob Agents Chemother **2009**; 53:1670–1672.
40. Strain MC, Gunthard HF, Havlir DV, et al. Heterogeneous clearance rates of long-lived lymphocytes infected with HIV: Intrinsic stability predicts lifelong persistence. Proc Natl Acad Sci U S A **2003**; 100: 4819–4824.
41. Allers K, Knoepfel SA, Rauch P, et al. Persistence of lamivudine-sensitive HIV-1 quasispecies in the presence of lamivudine in vitro and in vivo. J Acquir Immune Defic Syndr **2007**; 44:377–385.
42. Bergroth T, Ekici H, Gisslen M, et al. Selection of drug-resistant HIV-1 during the early phase of viral decay is uncommon in treatment-naive patients initiated on a three- or four-drug antiretroviral regimen including lamivudine. J Med Virol **2009**; 81:1–8.
43. Landman R, Descamps D, Peytavin G, et al. Early virologic failure and rescue therapy of tenofovir, abacavir, and lamivudine for initial treatment of HIV-1 infection: TONUS study. HIV Clin Trials **2005**; 6:291–301.
44. Gallant JE, Rodriguez AE, Weinberg WG, et al. Early virologic nonresponse to tenofovir, abacavir, and lamivudine in HIV-infected antiretroviral-naive subjects. J Infect Dis **2005**; 192:1921–1930.
45. Luber AD. Genetic barriers to resistance and impact on clinical response. Med Gen Med **2005**; 7:69.
46. von Wyl V, Yerly S, Boni J, et al. Emergence of HIV-1 drug resistance in previously untreated patients initiating combination antiretroviral treatment: a comparison of different regimen types. Arch Intern Med **2007**; 167:1782–1790.
47. Bierman WF, van Agtmael MA, Nijhuis M, Danner SA, Boucher CA. HIV monotherapy with ritonavir-boosted protease inhibitors: a systematic review. AIDS **2009**; 23:279–291.
48. Goodman DD, Margot NA, McColl DJ, Miller MD, Borroto-Esoda K, Svarovskaia ES. Pre-existing low-levels of the K103N HIV-1 RT mutation above a threshold is associated with virological failure in treatment-naive patients undergoing EFV-containing antiretroviral treatment. Antiviral Therapy **2009**; 14:A43 (abstract 1, XVIII International HIV Drug Resistance Workshop 2009).