Minority Quasispecies of Drug-Resistant HIV-1 That Lead to Early Therapy Failure in Treatment-Naive and -Adherent Patients

Karin J. Metzner,[1,a,b] Stefano G. Giulieri,[2,a] Stefanie A. Knoepfel,[1] Pia Rauch,[1] Philippe Burgisser,[3] Sabine Yerly,[4] Huldrych F. Günthard,[5] and Matthias Cavassini[2]

[1]Institute of Clinical and Molecular Virology, University of Erlangen-Nuremberg, Erlangen, Germany; [2]Infectious Diseases Service and [3]Laboratory of Immunology and Allergy, Centre Hospitalier Universitaire Vaudois and University of Lausanne, Lausanne, [4]Laboratory of Virology, Division of Infectious Diseases, University Hospital of Geneva, Geneva, and [5]Department of Medicine, Division of Infectious Diseases and Hospital Epidemiology, University Hospital Zurich, Zurich, Switzerland

Background. Early virological failure of antiretroviral therapy associated with the selection of drug-resistant human immunodeficienc virus type 1 in treatment-naive patients is very critical, because virological failure significantl increases the risk of subsequent failures. Therefore, we evaluated the possible role of minority quasispecies of drug-resistant human immunodeficienc virus type 1, which are undetectable at baseline by population sequencing, with regard to early virological failure.

Methods. We studied 4 patients who experienced early virological failure of a first-lin regimen of lamivudine, tenofovir, and either efavirenz or nevirapine and 18 control patients undergoing similar treatment without virological failure. The key mutations K65R, K103N, Y181C, M184V, and M184I in the reverse transcriptase were quantifie by allele-specifi real-time polymerase chain reaction performed on plasma samples before and during early virological treatment failure.

Results. Before treatment, none of the viruses showed any evidence of drug resistance in the standard genotype analysis. Minority quasispecies with either the M184V mutation or the M184I mutation were detected in 3 of 18 control patients. In contrast, all 4 patients whose treatment was failing had harbored drug-resistant viruses at low frequencies before treatment, with a frequency range of 0.07%–2.0%. A range of 1–4 mutations was detected in viruses from each patient. Most of the minority quasispecies were rapidly selected and represented the major virus population within weeks after the patients started antiretroviral therapy. All 4 patients showed good adherence to treatment. Nonnucleoside reverse-transcriptase inhibitor plasma concentrations were in normal ranges for all 4 patients at 2 separate assessment times.

Conclusions. Minority quasispecies of drug-resistant viruses, detected at baseline, can rapidly outgrow and become the major virus population and subsequently lead to early therapy failure in treatment-naive patients who receive antiretroviral therapy regimens with a low genetic resistance barrier.

The use of combination antiretroviral therapy (ART) has remarkably reduced the morbidity and mortality of subjects infected with HIV [1], but these benefit can be compromised by the development of drug resistance [2]. Since the firs reports of primary infection with drug-resistant HIV-1, transmission of drug-resistant HIV strains has been a growing concern [3–5].

Because primary infection with a resistant strain may decrease the efficac of initial therapy, resistance testing before initiation of ART, in the context of recent and even established HIV-1 infection, is recommended [6]. However, one major limitation of techniques such as population sequencing is the inability to detect drug-resistant minority quasispecies unless they represent 20%–25% of the total population [7]. Allele-specifi real-time PCR (AS-PCR) allows the detection of minority quasispecies with discriminatory abilities to de-

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[a] K.J.M. and S.G.G. contributed equally to this work.

[b] Present affiliation: Department of Medicine, Division of Infectious Diseases and Hospital Epidemiology, University Hospital Zurich, Zurich, Switzerland.

Reprints or correspondence: Dr. Karin J. Metzner, University Hospital Zurich, Dept. of Medicine, Div. of Infectious Diseases and Hospital Epidemiology, Rämistrasse 100, CH-8091 Zurich, Switzerland (Karin.Metzner@usz.ch).

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tect viral variants that represent as little as 0.01% of the population. Using this technique, we have shown that drug-resistant HIV variants could be detected in 20% of acute seroconverters; the drug-resistant virus population was detected by population sequencing in only one-half of those patients [8]. The clinical importance of minority quasispecies has yet to be determined. To date, only a few observations have shown that minority quasispecies of drug-resistant viruses can emerge as major virus populations after initiation of salvage therapy in pretreated patients [9–11].

Virological failure of ART significantl increases the risk of clinical progression and, when associated with the appearance of drug-resistant viruses, limits further treatment options [12, 13]. Therefore, early virological failure (eVF) within a few weeks after ART initiation in treatment-naive patients is especially troublesome. Several reasons have been identifie for eVF associated with HIV-1 drug resistance, such as poor adherence to treatment, drug combinations with low antiretroviral potencies, or pharmacokinetics issues (e.g., malabsorption and drug interactions) [14–17]. Here, we addressed the role of minority quasispecies of drug-resistant viruses involved in the eVF of treatment-naive patients. Four patients experienced eVF despite excellent adherence and adequate drug plasma levels during treatment with a potent first-lin nonnucleoside reverse-transcriptase inhibitor (NNRTI)–based regimen that was chosen after baseline resistance testing. Therefore, we conducted a case-control analysis of baseline and on-treatment samples by AS-PCR assays for reverse-transcriptase (RT) mutations K65R, K103N, Y181C, M184V, and M184I, and we compared the results with those of standard virtual phenotype analysis using population sequencing performed on the same samples.

METHODS

Study design and patients. All patients were treated at the University Hospital of Lausanne from March 2005 through August 2006. We identifie as case patients those who experienced eVF while receiving first-lin NNRTI-based regimen despite good adherence to treatment (as certifie by directly observed therapy or weekly community visits by a qualifie nurse), adequate antiretroviral drug plasma levels, and no HIV-1 drug resistance at baseline (determined by population sequencing). Control patients were selected retrospectively on the basis of characteristics as follows: no failure associated with first-lin NNRTI-based regimen within the firs 9 months of treatment, no resistance mutations at baseline, and HIV-1 subtypes and baseline viral loads comparable to those of the case patients. To confi m that case and control patients had no resistance mutations at baseline by population sequencing, 2 investigators performed a blind review of all fl orograms of the sequences. The baseline characteristics of case and control patients are summarized in tables 1 and 2, respectively. Plasma sampling took place before and during ART. All patients agreed to plasma sampling and resistance testing.

HIV quantificatio and resistance testing. Plasma HIV-1 RNA was quantifie using the Cobas AmpliPrep/Cobas TaqMan HIV-1 Test (Roche Diagnostics), with a detection limit of 40 HIV-1 RNA copies/mL of plasma. Resistance testing by population sequencing was performed by using VirtualPhenotype (Virco).

AS-PCR for the detection and quantificatio of minority quasispecies of K65R, K103N, Y181C, M184V, and M184I drug-resistant HIV-1 variants. Up to 2 blood samples obtained before the start of ART, all follow-up samples obtained until the start of second-line treatment for case patients, and 1 baseline sample from control patients were tested by AS-PCR assays, which were performed blind. Evaluation of discriminatory abilities and validation of the K103N and M184V AS-PCR assays were described elsewhere [8, 18]. AS-PCRs of the K65R, Y181C, and M184I mutations and their corresponding wild-type variants are described in the Appendix.

Statistical analysis. To investigate the significanc of differences in the presence of minority quasispecies at baseline in both groups of patients, statistical analysis was performed using the 2-tailed Fisher's exact probability test for binary data. P values $<.05$ were considered to be signif cant.

RESULTS

Case patients. Four treatment-naive patients received lamivudine, tenofovir, and nevirapine (3 patients) or efavirenz (1 patient) as their firs ART regimen. Treatment for patient 3 was switched from nevirapine to efavirenz after 8 weeks because of gastrointestinal adverse effects. Patient characteristics are summarized in table 1. For case patients, viral load at baseline had a range of 430,000–1,440,000 HIV-1 RNA copies/mL of plasma. Before initiation of ART, resistance testing was performed by population sequencing. No evidence of drug resistance was observed in any of the 4 patients (figu e 1). All patients were monitored with regard to plasma concentrations of the NNRTI. Measurement of plasma NNRTI concentration after $\geqslant$2 weeks of treatment and after 1–2 months showed values for efavirenz in the upper range in patient 2 and at the ~50th percentile for nevirapine in patient 3. Nevirapine concentrations were within normal ranges in patients 1 and 4. For all case patients, viral load decrease at 4–7 weeks had a range of 0–1 log, and viral load remained >100,000 HIV-1 RNA copies/mL of plasma until the start of second-line treatment. A second resistance test with use of standard genotyping was performed 9–27 weeks after starting the first-lin regimen (figu e 1). Viruses of all patients harbored multiple mutations that conferred resistance to lamivudine, to NNRTIs, and, partly, to tenofovir. Salvage therapy was initiated 15–31 weeks after the start of the fir t-line regimen. Second-line regimens included zidovudine (2 patients) or

Table 1. Characteristics of patients who experienced virological failure within the first weeks after starting first ART (case patients).

Patient	Sex	Route of infection	CDC classification	HIV-1 subtype	Baseline viral load, HIV-1 RNA copies/mL of plasma	First-line treatment	Viral load nadir		Salvage therapy		Duration of salvage therapy to achieve viral load, weeks	
							Week after initiation of first-line treatment	HIV-1 RNA, copies/mL of plasma	Start after initiation of first-line treatment, weeks	Second-line treatment	<400 HIV-1 RNA copies/mL of plasma	<40 HIV-1 RNA copies/mL of plasma
1	M	IVDA	C3	B	797,000	3TC, TDF, NVP	5	630,000	25	AZT, SQV, ATV	14	64
2	M	Het	B3	C	1,440,000	3TC, TDF, EFV	7	425,000	31	AZT, SQV, LPV	6	19
3	F	Het	B3	AG	510,000	3TC, TDF, NVP[a]	4	82,900	15	D4T, ATV, LPV	12	71
4	F	Het	C3	CRF02_AG	430,000	3TC, TDF, NVP	0	430,000[b]	15	3TC, D4T, SQV, ATV	10	20

NOTE. 3TC, lamivudine; ART, antiretroviral therapy; ATV, atazanavir (boosted with ritonavir); AZT, zidovudine; CDC, Centers for Disease Control and Prevention; D4T, stavudine; EFV, efavirenz; Het, heterosexual sex; IVDA, injection drug abuse; LPV, lopinavir (boosted with ritonavir); NVP, nevirapine; SQV, saquinavir; TDF, tenofovir.

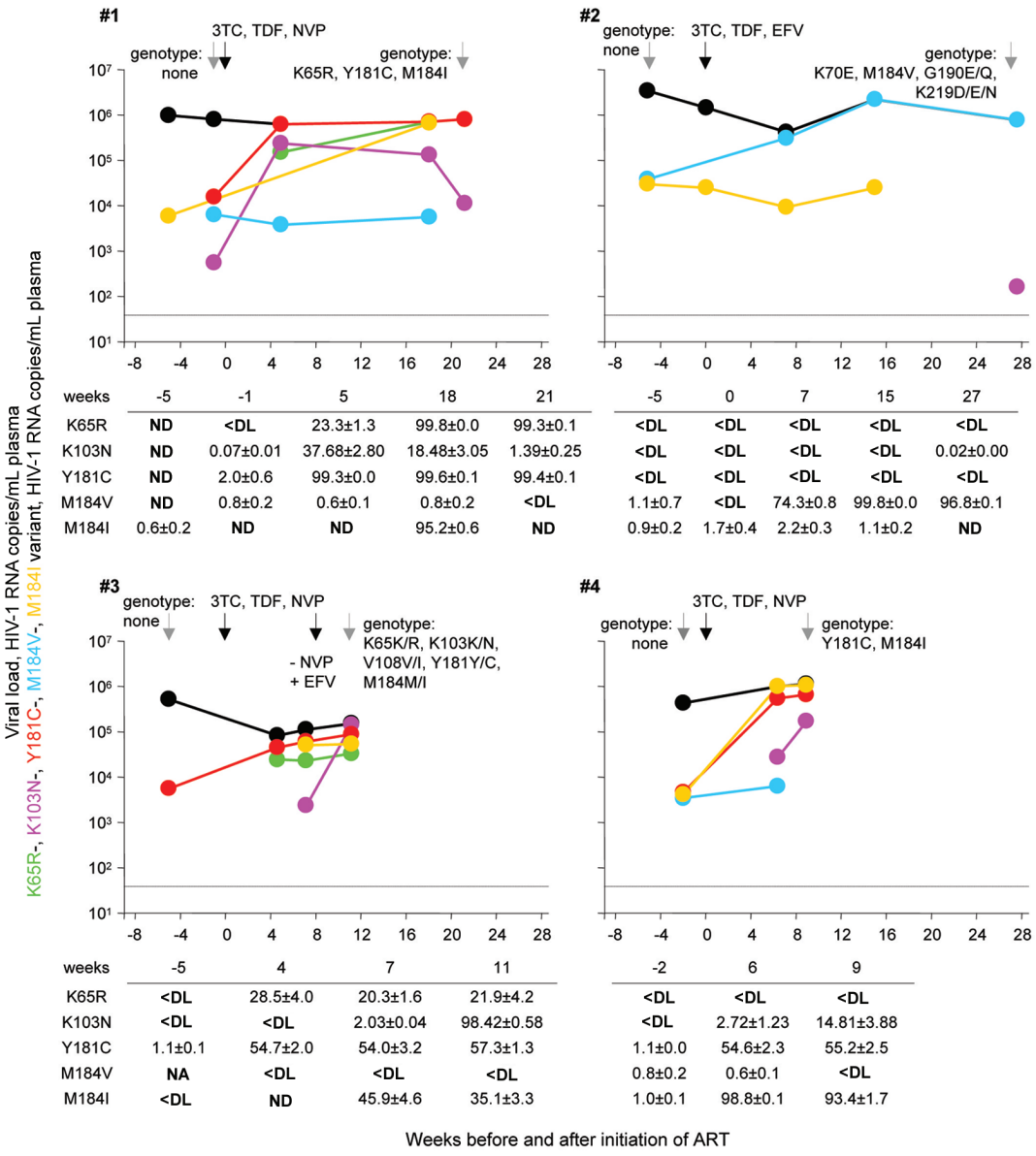
[a] Patient 3 switched to EFV after 8 weeks because of gastrointestinal adverse effects.

[b] Patient 4 never had a viral load decrease during first-line treatment.

Table 2. Characteristics of patients who did not experience virological failure within 9 months after starting first ART (control patients).

Patient	Sex	Route of infection	CDC classification	HIV-1 subtype	Baseline viral load, HIV-1 RNA copies/mL of plasma	Time to achieve viral load <40 copies/mL, weeks	Baseline RT resistance mutations, population sequence	First-line treatment	AS-PCR result ± SD at baseline, %				
									K65R	K103N	Y181C	M184V	M184I
5	F	Het	C3	F1	1,760,000	26	None	3TC, TDF, EFV	<DL	<DL	<DL	<DL	<DL
6	M	MSM	A2	B	34,300	5	None	3TC, TDF, EFV	<DL	<DL	<DL	<DL	ND
7	M	IVDA	B2	CRF13_CPX	31,400	14	None	3TC, TDF, EFV	<DL	<DL	<DL	<DL	ND
8	F	Het	A2	CRF14_BG	78,200	15	None	3TC, TDF, EFV	<DL	<DL	<DL	<DL	ND
9	M	Het	A2	CRF02_AG	14,900	21	None	3TC, TDF, EFV	<DL	<DL	<DL	14.1 ± 0.3	<DL
10	F	Het	B2	B	1,600,000	18	215D	3TC, TDF, EFV	<DL	<DL	<DL	<DL	1.0 ± 0.1
11	M	Het	A3	C	104,000	7	None	3TC, TDF, EFV	<DL	<DL	<DL	<DL	<DL
12	M	Het	C3	B	272,000	20	None	3TC, TDF, EFV	<DL	<DL	<DL	<DL	<DL
13	M	Unknown	A2	B	213,000	21	None	3TC, TDF, NVP	<DL	<DL	<DL	<DL	<DL
14	M	MSM	B3	B	496,000	41	None	3TC, TDF, EFV	<DL	<DL	<DL	<DL	<DL
15	F	Het	A2	AE	98,200	21	None	3TC, TDF, EFV	<DL	<DL	<DL	NA	<DL
16	M	MSM	B3	B	85,400	30	None	3TC, TDF, EFV	<DL	<DL	<DL	<DL	<DL
17	M	MSM	C3	B	813,000	12	None	3TC, TDF, EFV	<DL	<DL	<DL	<DL	2.1 ± 0.4
18	M	Het	A2	F1	183,000	27	None	3TC, TDF, EFV	<DL	<DL	<DL	<DL	<DL
19	M	Het	A2	CX	25,600	6	None	3TC, TDF, EFV	<DL	<DL	<DL	<DL	ND
20	M	IVDA	B2	B	139,000	17	None	3TC, TDF, EFV	<DL	<DL	<DL	<DL	<DL
21	M	Het	B2	B	90,900	9	None	3TC, TDF, EFV	<DL	<DL	<DL	<DL	<DL
22	F	IVDA	B3	B	435,000	12	None	3TC, TDF, EFV	<DL	<DL	<DL	<DL	<DL

NOTE. 3TC, lamivudine; ART, antiretroviral therapy; AS-PCR, allele-specific real-time PCR assay; CDC, Centers for Disease Control and Prevention; <DL, below detection limit; EFV, efavirenz; Het, heterosexual sex; IVDA, injection drug abuse; MSM, men who have sex with men; NA, not applicable (because of negative results for both wild-type and mutant sequences); ND, not determined; RT, reverse transcriptase; TDF, tenofovir.



#1 weeks	-5	-1	5	18	21
K65R	ND	<DL	23.3±1.3	99.8±0.0	99.3±0.1
K103N	ND	0.07±0.01	37.68±2.80	18.48±3.05	1.39±0.25
Y181C	ND	2.0±0.6	99.3±0.0	99.6±0.1	99.4±0.1
M184V	ND	0.8±0.2	0.6±0.1	0.8±0.2	<DL
M184I	0.6±0.2	ND	ND	95.2±0.6	ND

#2	-5	0	7	15	27
K65R	<DL	<DL	<DL	<DL	<DL
K103N	<DL	<DL	<DL	<DL	0.02±0.00
Y181C	<DL	<DL	<DL	<DL	<DL
M184V	1.1±0.7	<DL	74.3±0.8	99.8±0.0	96.8±0.1
M184I	0.9±0.2	1.7±0.4	2.2±0.3	1.1±0.2	ND

#3 weeks	-5	4	7	11
K65R	<DL	28.5±4.0	20.3±1.6	21.9±4.2
K103N	<DL	<DL	2.03±0.04	98.42±0.58
Y181C	1.1±0.1	54.7±2.0	54.0±3.2	57.3±1.3
M184V	NA	<DL	<DL	<DL
M184I	<DL	ND	45.9±4.6	35.1±3.3

#4	-2	6	9
K65R	<DL	<DL	<DL
K103N	<DL	2.72±1.23	14.81±3.88
Y181C	1.1±0.0	54.6±2.3	55.2±2.5
M184V	0.8±0.2	0.6±0.1	<DL
M184I	1.0±0.1	98.8±0.1	93.4±1.7

Figure 1. Kinetics of viral load and quantification of minority quasispecies of drug-resistant viruses in patients who received their first antiretroviral therapy (ART) and experienced early virological failure within the first weeks. HIV-1 RNA in plasma was measured using the Cobas AmpliPrep/Cobas TaqMan HIV-1 Test, with a lower limit of 40 copies/mL of plasma *(black circles).* Genotype data are depicted *(gray arrows)* and were obtained by population sequencing with use of the VirtualPhenotype analysis; "none" means that no mutations associated with drug resistance were detected in the reverse transcriptase [19]. Quantification of drug-resistant variants carrying the K65R, K103N, Y181C, M184V, or M184I mutation and drug-susceptible viruses was performed by allele-specific PCR. The percentage of the virus population carrying the specific mutation was used to calculate the absolute HIV-1 RNA copies/mL of plasma of the drug-resistant quasispecies on the basis of the corresponding total viral load measurement. Copy numbers representing the K65R variant *(green circles),* K103N variant *(pink circles),* Y181C variant *(red circles),* M184V variant *(blue circles),* and M184I variant *(yellow circles)* are shown. The thin black line indicates the limit of the viral load and allele-specific PCR measurements (40 HIV-1 RNA copies/mL of plasma). Percentages (± SD) of each drug-resistant virus population are given. 3TC, lamivudine; <DL, below detection limit; EFV, efavirenz; NA, not applicable (because of negative results for both wild-type and mutant sequences); ND, not determined; NVP, nevirapine; TDF, tenofovir.

stavudine (2 patients) in combination with 2 boosted protease inhibitors. After 6–14 weeks of salvage therapy, all patients reached a viral load <400 HIV-1 RNA copies/mL of plasma. Undetectable levels (<40 HIV-1 RNA copies/mL of plasma) were achieved in all patients after 19–71 weeks of salvage therapy (table 1).

Retrospectively, the key resistance mutations K65R, K103N, Y181C, M184V, and M184I within the RT were further analyzed

by sensitive AS-PCR, which allowed the quantificatio of minority quasispecies of drug-resistant viruses. In all 4 patients, 1–4 drug resistance mutations were detected at frequencies of 0.07%–2.0% before initiation of first-lin ART. The K103N mutation was found in 1 patient; the Y181C and the M184V or M184I variants were found in 3 patients (figu e 1). Rapid selection of those variants and the additional appearance of 1 or 2 of those mutations, which were not detectable at baseline, were observed in all patients.

In patient 1, the K103N, Y181C, M184V, and M184I mutations were detected as minority quasispecies at low frequencies, with a range (± SD) of 0.07% ± 0.01% to 2.0% ± 0.6% at baseline (figu e 1). Five weeks after starting ART, 99.3% ± 0.0% of viruses already carried the Y181C mutation, which remained at those levels through week 21. The K103N mutation was temporarily selected; ~37.7% of viruses harbored the K103N mutation at week 5, 18.5% of viruses harbored the mutation at week 18, and this frequency further decreased at week 21, to 1.39% ± 0.25% (figu e 1), which suggests that the Y181C mutation alone was sufficien to confer resistance against nevirapine. The K65R mutation was not detected before ART; however, 23.3% ± 1.3% of viruses already harbored the K65R mutation at week 5. This frequency increased to levels >99% at weeks 18 and 21. Both the M184V and the M184I mutations were present in similar frequencies before ART was initiated. The M184V mutation remained at low frequencies (<1% at weeks 5 and 18), and the M184I mutation was selected and was represented in 95.2% ± 0.6% of the virus population at week 18. The K65R, Y181C, and M184I mutations were also detected by population sequencing at week 21 (f gure 1).

In patient 2, the M184V and the M184I mutations were present at baseline as minor variants at similar frequencies. For this patient, the M184V mutation was consequently selected during therapy failure, and the M184I mutation remained a minority quasispecies at frequencies of 1%–2%. The K65R, K103N, and Y181C mutations were not detectable as minority quasispecies before ART. In addition, none of those mutations were developed and selected in this patient. Of those 3 mutations, only the K103N mutation was detected, at very low levels, at week 27 (0.02% ± 0.00%). The presence of the M184V mutation as a major virus population was confi med by population sequencing. For this patient, other mutations (K70E, G190E/Q, and K219D/E/N)—in addition to the M184V mutation—led to eVF (fi ure 1).

In patient 3, the Y181C mutation already represented in 1.1% ± 0.1% of viruses before ART, increased to 54.7% ± 2.0% at week 4, and remained at this level during the following 7 weeks. The K103N mutation was detectable for the fi st time at week 7, at a frequency of 2.03% ± 0.04%, and increased rapidly during the following 4 weeks, to 98.42% ± 0.58%, after the switch from nevirapine to efavirenz at week 8. The K65R mutation was undetectable at baseline; however, 28.5% ± 4.0% of viruses harbored this mutation at week 4. No further selection was observed; the K65R mutation remained at frequencies of 20%–22% at weeks 7 and 11. A similar pattern was observed with regard to the M184I mutation. This variant was undetectable at baseline and represented 45.9% ± 4.6% of the virus population at week 7 and represented 35.1% ± 3.3% at week 11. The M184V mutation was never detectable in this patient.

In patient 4, after initiation of ART, we also observed a rapid selection of the Y181C mutation, which was already present at baseline at a frequency of 1.1% ± 0.0%. In addition, the K103N mutation was selected and was represented in 14.81% ± 3.88% of viruses at week 9, although it had not been detected at baseline. As was observed in patient 1, viruses containing the M184I mutation (which was present for patient 4 at baseline at a frequency of 1.0% ± 0.1%) were selected as a major virus population despite the presence of the M184V mutation as a minority quasispecies at weeks −2 and 6 (0.8% ± 0.2% and 0.6% ± 0.1%, respectively) (figu e 1). Thus, mutations that confer resistance against lamivudine and nevirapine were rapidly selected, but no mutation associated with resistance to tenofovir was identifie in this patient.

Control patients. Control patients suppressed virus replication to undetectable levels within 5–41 weeks after treatment initiation (table 2). Genotypic resistance testing by population sequencing was performed for all control patients before ART initiation. No mutations associated with drug resistance were identified None of the mutations K65R, K103N, and Y181C were present as minority quasispecies in the control group. Control patients 10 and 17 harbored the M184I mutation (at frequencies of 1.0% ± 0.1% and 2.1% ± 0.4%, respectively). In patient 9, 14.1% ± 0.3% of viruses harbored the M184V mutation before ART initiation (table 2); however, this did not lead to eVF.

Therefore, only 3 of 18 patients who efficientl suppressed virus replication after starting ART harbored minority quasispecies of viruses that carried either the M184V or the M184I mutation at baseline. In contrast, 1–4 drug resistance mutations were present as minority quasispecies at baseline in all 4 patients who experienced eVF. With use of the 2-tailed Fisher's exact probability test for the calculation of significance those differences are significan on the basis of numbers of patient with and without detectable minority quasispecies of drug-resistant HIV-1 at baseline (3 of 18 patients vs. 4 of 4 patients; $P = .005$).

DISCUSSION

Although the number of treatment-naive patients who experienced eVF within just weeks after starting ART is, fortunately, low [20], eVF is particularly problematic for the patient and

the physician, especially when poor adherence can be excluded as the reason for eVF. Further reasons for nonresponse to first-line ART despite good adherence are currently not well understood. Here, we show that the presence of drug-resistant viruses in low frequencies at baseline—that is, those not detected by conventional genotypic testing—is associated with eVF, because of rapid selection of those drug-resistant viruses in 4 of 4 patients, despite well-documented, excellent adherence and adequate drug plasma levels. We exclude nonadherence as the reason for eVF among our patients for several reasons. Two of 4 patients received directly observed therapy. One patient, who lived in a refugee home, had weekly community surveillance of drug intake by a nurse who used a weekly pill rack. Drug concentrations in plasma were within normal ranges in all 4 patients. In addition, all patients showed an optimal (>95%) adherence to the second-line regimen, which was surveyed by medication event–monitoring systems.

Several studies have shown that minority quasispecies of drug-resistant viruses can be found despite negative results of standard genotyping. This was clearly demonstrated in women after treatment with single-dose nevirapine to prevent mother-to-child transmission of HIV-1 [21, 22], as well as in acute seroconverters [8], patients undergoing structured treatment interruptions [18], and patients experiencing virological failure [10]. However, the clinical implications of minority quasispecies of drug-resistant viruses are still unclear. Some observations suggest that minority quasispecies of drug-resistant viruses can emerge as major virus populations after initiation of salvage therapy in pretreated patients [9–11, 23], but so far, only scarce evidence exists that the minority quasispecies can affect the outcome of first-line therapy. One case report demonstrated the emergence of minority quasispecies of drug-resistant viruses in a previously treatment-naive patient who initiated ART; however, several other drug resistance mutations were detected by population sequencing at baseline in this patient, which suggests that drug-resistant HIV-1 had been transmitted [24]. Our own data from the Primary HIV-1 Infection Cohort in Zurich, Switzerland, and a study in France showed no significant difference between the outcome of first-line therapy in acutely or recently HIV-1 infected patients who carried minority quasispecies of drug-resistant viruses and those who did not [25, 26]. In contrast to our current study, most of those patients received 2 nucleoside reverse-transcriptase inhibitors and a boosted protease inhibitor and, thus, ART regimens with high genetic resistance barriers. Recently, Johnson et al. [27] showed a correlation between the presence of minority quasispecies of drug-resistant viruses at baseline and virological failure in treatment-naive patients who received efavirenz-lamivudine and abacavir or zidovudine. Standard genotyping after treatment failure revealed the selection of those variants in 4 of 7 patients, which supports our results. However, no detailed information is available with regard to adherence to treatment by patients in that study. In a previous study [28], we observed a rapid selection of drug-resistant HIV-1 variants in patients who showed a slow decrease in viral load after starting their first ART, independent of preexisting minority quasispecies of drug-resistant viruses. However, few patients were treated with an NNRTI, and the regimen was intensified within weeks for all patients who experienced a slow decrease in viremia.

We show for the first time, to our knowledge, that patients can experience eVF despite good adherence; no evidence, by population sequencing, of transmission of drug-resistant HIV-1; and a potent ART regimen, as evidenced by the presence of minority quasispecies of drug-resistant viruses before the start of ART and subsequent rapid selection of those variants. Although the number of patients was small and the study was performed retrospectively, all patients were treated with a similar regimen and during the same time period. One limitation of our study is the small proportion of nevirapine-based regimens used to treat control patients. The issue of a higher efficacy of efavirenz over nevirapine is still a matter of debate [29]. However, the largest randomized, controlled trial that compared efavirenz with nevirapine (2NN study [30]) did not reveal a statistically significantly higher virological failure rate in the nevirapine arm. In addition, the AS-PCR analyses were performed blind. Therefore, our data strongly support the hypothesis that minority quasispecies of drug-resistant viruses have clinical implications in certain settings. In this context, it has to be emphasized that all 4 patients were treated with a first-line regimen that was characterized by low genetic barrier to resistance. Especially with regard to first-line regimens containing efavirenz or nevirapine, it might be possible to identify patients at risk of failure by more-sensitive methods, such as AS-PCR.

The pattern of drug-resistant minority quasispecies found in our patients deserves another important consideration. The impact of minority quasispecies of drug-resistant viruses at baseline appears to be dependent on the specific mutation detected. Both the M184V and the M184I mutations were found at baseline at similar frequencies in 3 patients who experienced eVF. Interestingly, the M184V mutation was selected in 1 of those patients, whereas the M184I mutation was rapidly selected in 2 other patients and contributed to eVF. This is in contrast with the observation that viruses harboring the M184V mutation are more replication competent than are viruses harboring the M184I mutation [31]. Moreover, the M184V and M184I mutations were also found in 3 control patients for whom the presence of those mutations at baseline did not lead to eVF. This shows that a mutation at codon 184 of the RT alone is not necessarily associated with therapy failure and suggests that the addition of other mutations in drug-resistant minority quasispecies may be required to develop failure [32].

In contrast, all 3 patients who harbored minority quasispecies with the Y181C mutation rapidly selected this virus population, which led to eVF. We found the K103N mutation at baseline in 1 patient, but the frequency remained low, and only the Y181C mutation was detected by conventional genotyping performed 21 weeks after initiation of firs ART. The selection of the Y181C mutation rather than K103N variants may be explained by the regimen, which contained nevirapine in these 3 patients. Nevirapine has been associated more frequently with the selection of the Y181C mutation in patients who experience failure of ART [2]. Taken together, these observations suggest that drug-resistant quasispecies have different implications with regard to the dynamics of virological failure. The Y181C mutation appears to have a major role, whereas the presence of the M184V mutation or the M184I mutation does not necessarily lead to virological failure. This is consistent with clinical observations and is probably related to the fitnes characteristic of the mutations [33]. In addition, the number or composition of different drug resistance mutations that are detectable as minority quasispecies at baseline might be important.

Notwithstanding great advances in the treatment of HIV infection and the availability of increasing possibilities for drug combinations, virological success of the first-lin regimen remains crucial for a good long-term prognosis [12, 13]. By using AS-PCR, we were able to show a rapid outgrow of minority quasispecies of drug-resistant viruses, undetected at baseline by conventional genotyping, that led to eVF despite excellent adherence and a potent standard regimen of lamivudine, tenofovir, and either efavirenz or nevirapine. Further studies to confi m the clinical benefi of the detection of minority quasispecies of drug-resistant viruses before starting ART in treatment-naive patients are warranted, especially in the context of ART regimens with low genetic barriers to resistance.

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APPENDIX

ALLELE-SPECIFIC REAL-TIME PCR (AS-PCR) FOR THE DETECTION AND QUANTIFICATION OF MINORITY QUASISPECIES OF K65R, Y181C, AND M184I DRUG-RESISTANT HIV-1 VARIANTS

AS-PCRs of the K65R, Y181C, and M184I mutations and their corresponding wild-type variants were designed and tested following the same procedures as described elsewhere [8, 18]. Plasma sampling, HIV-1 RNA isolation, and viral cDNA synthesis and amplificatio were performed as described elsewhere [8, 18]. In brief, 4 separate short-cycle firs PCRs were performed to optimize the primer binding sites with use of the following primers: (1) K103 EP1 [8], M184 EP1 [18], K103 EP2 [8], and M184 EP2 [18] in a multiplex PCR; (2) K65 EP1 5′-ACTCCAGTATTTGCCATAAAG-3′ (nucleotides [nt] 2721–2741; nt positions are based on the reference strain HXB2) and K103 EP2 [8]; (3) Y181 EP1 5′-TCAGTACAATGTGCTTCCAC-3′ (nt 2981–3000) and Y181 EP2 5′-CATACAAATCATCCATGTATTG-3′ (nt 3′093–3114); and (4) [M184I EP1 5′-TATCAGTACAATGTGCTTCCAC-3′ (nt 2979–3000) and M184I EP2 5′-GTCAGATCCTACATACAAATCAT-3′ (nt 3103–3125). PCR conditions and amplicon purificatio are described elsewhere [8, 18].

AS-PCR was performed using the purifie PCR products and the primers IN K65 5′-TCCAGTATTTGCCATAAAGIA-3′ (nt 2723–2743) or IN K65R 5′-CCAGTATTTGCCATAAAGIG-3′ (nt 2724–2743) and pol 3002 rc 5′-CTGTGGAAGCACATTGTACTG-3′ (nt 2812–2835) for the K65R AS-PCR, IN Y181 5′-CATACAAATCATCCATGTATTGIT-3′ (nt 3091–3114) or IN Y181C 5′-ATACAAATCATCCATGTATTGIC-3′ (nt 3091–3115) and pol 2981 5′-TCAGTACAATGTGCTTCCACAGG-3′ (nt 2981–3003) for the Y181C AS-PCR, and IN M184wt 5′-AGATCCTACATACAAATCATIC-3′ (nt 3101–3122) or IN M184I 5′-CAGATCCTACATACAAATCATIT-3′ (nt 3101–3123) and pol 2981 for the M184I AS-PCR. Further details about the PCRs and data analysis are described elsewhere [8, 18]. Each AS-PCR assay has a detection limit of 10 HIV-1 DNA copies per reaction, with a linear dynamic range of >6 logs. The estimated discriminatory abilities of the different drug-susceptible and drug-resistant sequences are as follows: 0.01% for detection of the K103N mutation; 0.2% for detection of the Y181C, M184V, and M184I mutations; and 0.4% for detection of the K65R mutation as minority quasispecies. For each patient sample, an individual cutoff was estimated on the basis of the viral load as described elsewhere, because low viral loads diminish the discriminatory ability of each assay [18]. Nonspecif c amplif cation by AS-PCR with use of SYBR green for detection of double-stranded DNA was excluded by melting-curve analysis.

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