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Low-frequency drug-resistant HIV-1 and risk of virological failure to first-line NNRTI-based ART: a multicohort European case–control study using centralized ultrasensitive 454 pyrosequencing

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Objectives: It is still debated if pre-existing minority drug-resistant HIV-1 variants (MVs) affect the virological outcomes of first-line NNRTI-containing ART.

Methods: This Europe-wide case–control study included ART-naive subjects infected with drug-susceptible HIV-1 as revealed by population sequencing, who achieved virological suppression on first-line ART including one NNRTI. Cases experienced virological failure and controls were subjects from the same cohort whose viraemia remained suppressed at a matched time since initiation of ART. Blinded, centralized 454 pyrosequencing with parallel bioinformatic analysis in two laboratories was used to identify MVs in the 1%–25% frequency range. ORs of virological failure according to MV detection were estimated by logistic regression.

Results: Two hundred and sixty samples (76 cases and 184 controls), mostly subtype B (73.5%), were used for the analysis. Identical MVs were detected in the two laboratories. 31.6% of cases and 16.8% of controls harboured pre-existing MVs. Detection of at least one MV versus no MVs was associated with an increased risk of virological failure (OR = 2.75, 95% CI = 1.35–5.60, $P = 0.005$); similar associations were observed for at least one MV versus no NRTI MVs (OR = 2.27, 95% CI = 0.76–6.77, $P = 0.140$) and at least one MV versus no NNRTI MVs (OR = 2.41, 95% CI = 1.12–5.18, $P = 0.024$). A dose–effect relationship between virological failure and mutational load was found.

Conclusions: Pre-existing MVs more than double the risk of virological failure to first-line NNRTI-based ART.

Keywords: minority drug-resistant HIV-1 variants, CHAIN, antiretroviral therapy, European multicentre study

Introduction

Antiretroviral drug resistance testing is one of the mainstays of the clinical management of HIV-1 infection[1] and is essential to inform public health strategies to combat the HIV/AIDS epidemic.[2] Population sequencing of plasma viruses only detects the most abundant viral variants infecting each subject.[3,4] Thereby, clinicians might miss potentially relevant information when making treatment decisions and public health estimations underestimate the burden of HIV-1 drug resistance. Next-generation sequencing platforms detect minority viral variants with higher sensitivity, but the clinical relevance of this is under discussion.

NNRTIs are often prescribed as first-line ART as efavirenz is a recommended third drug in all treatment guidelines and nevirapine is one of the most affordable drugs in resource-limited settings.[5–8] These drugs are highly efficacious, but have a low genetic barrier to resistance; even HIV-1 with a single mutation can become fully resistant to NNRTIs.[1] The small impact of most NNRTI resistance mutations on HIV-1 replicative capacity allows their persistence in the virus population of the infected subjects[9] and their transmission to newly infected subjects. Using population sequencing, between 5% and 15% of new HIV-1 infections in Europe and the USA occur with an NNRTI-resistant virus.[10,11] An additional fraction of ART-naive subjects harbour minority drug-resistant variants, which could also be transmitted or be spontaneously generated in the absence of ART exposure.[12–14]

The best available evidence of the clinical relevance of minority NNRTI-resistant HIV-1 variants to date is a systematic review and pooled analysis of 10 published studies including 1263 ART-naive subjects who initiated first-line NNRTI-containing ART with drug-susceptible HIV-1 according to population sequencing.[15] In that study, detection of pre-existing minority drug-resistant HIV-1 variants (MVs) more than doubled the risk of virological failure to first-line ART, showing a direct dose–effect relationship between the level of MVs and the risk of virological failure. This analysis, however, pooled studies with heterogeneous designs, different MV detection techniques and inconsistent results.

To further clarify the impact of MVs on the virological outcomes of first-line NNRTI-containing ART in the light of these previous data, we designed a large European case–control study including standard operating procedures for data collection, centralized cDNA synthesis and 454 pyrosequencing and duplicate 454 bioinformatic analysis of MVs, all blinded for clinical outcomes.

Methods

Study objectives

This study sought to investigate the ability of a single, pre-ART, ultrasensitive HIV-1 genotypic test using 454 pyrosequencing (454 Life Sciences/Roche Diagnostics) to predict virological failure to first-line ART including two NRTIs and one NNRTI. Additional objectives were to evaluate the consistency of the observations across different subsets of subjects and to assess the presence of a dose–response relationship between mutational load and risk of virological failure.

Study design and participants

This was a case–control study nested within seven prospective European HIV-1 observational cohorts: Swiss HIV Cohort Study (SHCS), EuroSIDA Study in EuroCoord (EuroSIDA), Italian Cohort of Antiretroviral-Naive Patients (ICONA), German Competence Network (KompNet), Arevir Platform, ANRS CO3 Aquitaine Cohort (Aquitaine) and Royal Free Hospital (RFH) cohort. Clinical data were extracted from these cohorts using a standard operating procedure (see the Supplementary data available at *JAC* Online).

The study population included ART-naive subjects initiating ART with two NRTIs plus one NNRTI and achieving a viral load ≤50 copies/mL within the first 6 months following ART initiation. In addition, subjects had to have one stored plasma sample with HIV-1 RNA levels ≥10 000 copies/mL within 6 months prior to ART initiation and ≥3 months of virological follow-up after achieving virological suppression. Subjects with any pre-ART IAS-USA (March 2013)[1] NNRTI or NRTI resistance mutation detected by population sequencing or with NNRTI/NRTI mutations at a frequency >25% by 454 pyrosequencing were excluded.

Cases were subjects developing virological failure, defined as two consecutive HIV-1 RNA measurements >200 copies/mL after achieving HIV-1 RNA ≤50 copies/mL. Virological failure had to occur while the person was still receiving the initial NNRTI therapy. The first of the two HIV-1 RNA values >200 copies/mL was taken as time of the event. Controls were subjects selected from the same cohort whose HIV-1 RNA was still ≤200 copies/mL at a time matching the time of virological failure for the corresponding case. A minimum of two matched controls were chosen for every case. We applied the concept of case–control studies of dynamic populations for our case–control sampling;[16] thus, the same patient can be included in several case–control sets.

Plasma sample processing, RT–PCR and 454 pyrosequencing

Archived plasma samples with HIV-1 RNA levels ≥10 000 copies/mL and collected from the different cohorts within 6 months prior to ART initiation were shipped to Utrecht Medical Centre (Utrecht, The Netherlands), where cDNA was generated using random hexamers as primers. cDNAs were then shipped to the Institut für Immunologie und Genetik (Kaiserslautern, Germany), where 454 amplicons were immediately prepared and pyrosequenced in batches of 80 samples each in a 454 FLX Genome Sequencer using Titanium chemistry. Amplicons used for this analysis included amplicons A (protease), B, C and D (reverse transcriptase) from the 454/Roche HIV-1 Genotyping Kit v2 plus one additional amplicon designed specifically for this study (amplicon E), which covered the main NNRTI mutations (Figure S1). Of note, amplicon A was amplified but not sequenced, as protease data were considered not relevant for the present study. cDNA synthesis and 454 processing were performed blinded for clinical outcomes.

Data analysis

454 pyrosequencing sff files were analysed in parallel at the IrsiCaixa AIDS Research Institute (Badalona, Catalonia, Spain) and University Hospital Zurich (Zurich, Switzerland), blinded for clinical outcomes. Sequences were demultiplexed using both 5′ and 3′ multiple identifier barcodes. Roche's proprietary Amplicon Variant Analyzer software version 2.7 was used to call drug resistance mutations, based on the consensus alignment information for each sample, using the HIV-1 HXB2 clonal sequence (GenBank ID: K03455.1) as reference. A variant list containing all IAS-USA drug resistance mutations[1] was used. Mutants had to be well balanced (their frequency in forward and reverse reads had to be comprised within one log ratio) and to have been identified from a total of ≥300 reads to be considered evaluable; otherwise, the codon was considered WT. A sample was defined to have a detected MV if 1%–25% of the virus population showed that variant. The analyses of both centres showed identical results (Figure S2).

The prevalence of detected MVs in cases and controls was compared using the χ^2 test. Univariable and multivariable logistic regression analyses were used to estimate ORs of virological rebound according to the

prevalence of MVs. Separate models were constructed for: (i) the presence of at least one IAS-USA 2013 reverse transcriptase inhibitor (RTI) MV; (ii) the presence of at least one IAS-USA 2013 NRTI MV; (iii) the presence of at least one IAS-USA 2013 NNRTI MV; and (iv) mutational load. The latter was calculated by multiplying the mutant frequency in the virus population by the HIV-1 RNA levels detected in the same sample and expressed as mutant copy number/mL. If more than one MV was detected in the same subject, the exposure variable 'mutational load' was calculated by adding the copy number/mL of all mutants detected. For the analysis of risk, mutational load was evaluated as a categorical variable, i.e. 0, 400–1000 and >1000 copies/mL, cut-offs chosen *a priori* on the basis of previously published results.[15] All multivariable estimates were adjusted for factors included in the multivariable models shown in Table 2. Additional sensitivity analyses were performed by including 245 unique samples (each patient was included in the analysis only once) and after excluding sequences containing aligned in-frame stop codons.

Results

Subjects' disposition

Clinical data were obtained from a total of 3355 ART-naive patients from seven prospective European HIV-1 observational cohorts, all started ART containing two NRTIs and efavirenz or nevirapine, achieving viral suppression ≤50 HIV-1 RNA copies/mL plasma, and providing a genotypic HIV-1 drug resistance test prior to first-line ART, leading to the extraction of 366 patients (Figure 1): 12 from Arevir, 33 from Aquitaine, 14 from EuroSIDA, 75 from ICONA, 31 from KompNet, 78 from the RFH cohort and 123 from the Swiss HIV Cohort Study. One hundred and twenty-one (33%) samples were excluded from downstream analyses due to the following reasons: cDNA synthesis failed in 11 (3%) samples; 454 sequencing failed in 22 (6%) samples; NNRTI mutations were found at ≥25% frequency by 454 pyrosequencing in 23 (6%) pre-ART samples; demographic data could not be provided by the cohort in 5 (1%) samples; and an additional 60 (16%) had to be excluded due to incomplete case–control sets. The 245 included and 116 excluded patients (for whom demographic data were available) showed no differences in terms of age, gender, ethnicity and mode of transmission. In contrast, viral load was significantly lower in the excluded patients compared with the included patients [4.77 (4.42–5.11) versus 4.91 (4.58–5.32), median (IQR), $\log_{10}$ HIV-1 RNA copies/mL plasma, $P=0.003$]. This was caused by the fact that cDNA synthesis or 454 pyrosequencing failed predominantly in those samples with lower viral loads.

Applying the concept of case–control studies of dynamic populations for our case–control sampling,[16] i.e. allowing the same person/sample to be used as control for more than one case, 260 samples (76 cases and 184 matched controls) were analysed. There were 44 cases with two matched controls and 32 cases with three matched controls. In a sensitivity analysis, we used the 245 unique samples.

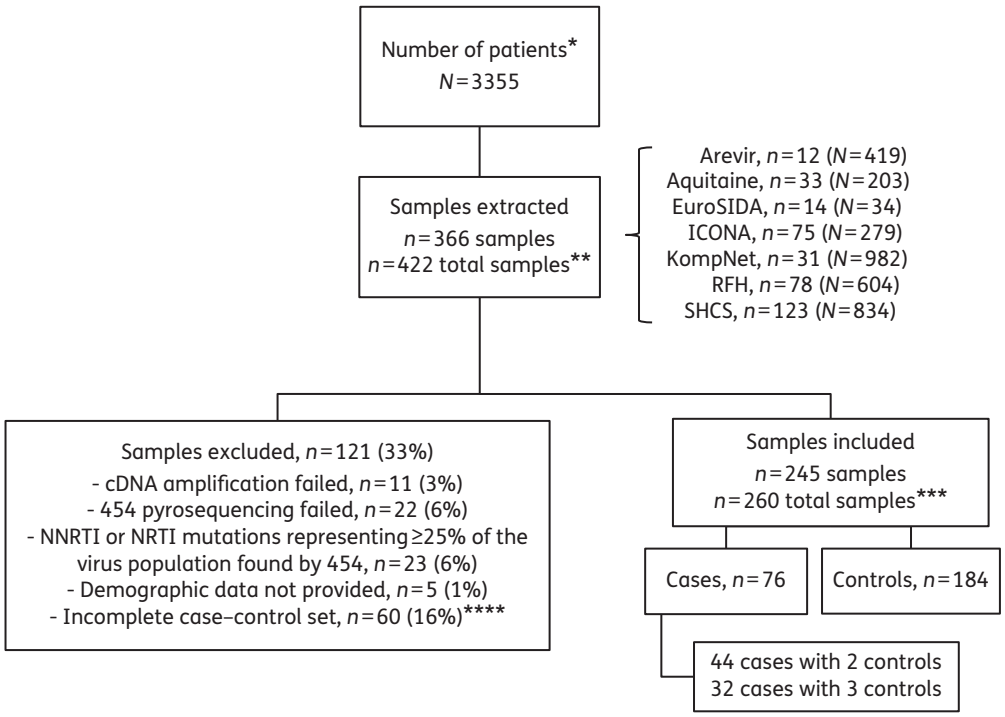

Figure 1. Subject disposition and scheme of the study design. MVs were analysed by next-generation sequencing in plasma samples with HIV-1 RNA levels ≥10 000 copies/mL collected within 6 months prior to ART initiation. *ART-naive patients in the cohorts starting ART containing two NRTIs and one NNRTI (efavirenz or nevirapine), achieving viral suppression ≤50 HIV-1 RNA copies/mL plasma and providing a genotypic HIV-1 drug resistance test prior to first-line ART. **Three hundred and sixty-six samples from 366 different patients were extracted from the different cohorts. Since several patients were matched controls in more than one case–control set, the total number of samples was 422. ***The main analyses were performed on 260 samples; a sensitivity analysis was performed on 245 unique samples. ****The exclusion of 61 patients due to technical challenges, NNRTI or NRTI mutations representing ≥25% of the virus population or missing demographic data led to the exclusion of an additional 60 samples since certain case–control sets were subsequently incomplete.

Table 1. Baseline characteristics of study population according to detection of MVs in plasma samples collected prior to first-line NNRTI-based ART

	≥1 IAS MV, *N*=55	No MVs, *N*=205	*P*	Total, *N*=260
Age (years)			0.776	
median (IQR)	36 (26–41)	33 (24–43)		34 (24–42)
Gender, *n* (%)			0.050	
female	6 (10.9)	47 (22.9)		53 (20.4)
Ethnicity, *n* (%)			0.143	
white	39 (70.9)	131 (63.9)		170 (65.4)
black	3 (5.5)	6 (2.9)		9 (3.5)
Asian	2 (3.6)	5 (2.4)		7 (2.7)
other	11 (20.0)	63 (30.7)		74 (28.5)
Mode of HIV transmission, *n* (%)			0.382	
MSM	27 (49.1)	77 (37.6)		104 (40.0)
heterosexual	13 (23.6)	82 (40.0)		95 (36.5)
intravenous drug use	6 (10.9)	26 (12.7)		32 (12.3)
other/unknown	9 (16.4)	20 (9.8)		29 (11.2)
HIV-1 subtype, *n* (%)			0.276	
A	1 (1.8)	7 (3.4)		8 (3.1)
B	40 (72.7)	151 (73.7)		191 (73.5)
C	3 (5.5)	10 (4.9)		13 (5.0)
D	0 (0.0)	1 (0.5)		1 (0.4)
F	2 (3.6)	3 (1.5)		5 (1.9)
G/J	3 (5.5)	7 (3.4)		10 (3.8)
CRF01_AE	0 (0.0)	9 (4.4)		9 (3.5)
CRF01_AG	4 (7.3)	6 (2.9)		10 (3.8)
Calendar year of starting NNRTI			0.250	
median (IQR)	2004 (2002–06)	2003 (2003–06)		2003 (2002–06)
Time from sample to ART initiation (months)			0.310	
median (IQR)	2 (0–4)	2 (0–4)		2 (0–4)
CD4+ count at ART initiation (cells/mm^3)			0.717	
median (IQR)	261 (196–326)	258 (173–371)		259 (176–353)
HIV-1 RNA at ART initiation ($\log_{10}$ copies/mL)			0.995	
median (IQR)	4.92 (4.55–5.36)	4.90 (4.49–5.32)		4.91 (4.58–5.33)
NNRTI started, *n* (%)			0.962	
nevirapine	9 (16.4)	33 (16.1)		42 (16.2)
efavirenz	46 (83.6)	172 (83.9)		218 (83.8)
NRTI pair started, *n* (%)			0.040	
recommended: ABC/3TC or TDF/FTC	19 (34.5)	62 (30.2)		81 (31.2)
alternative: ZDV/3TC or TDF/3TC	26 (47.3)	106 (51.7)		132 (50.8)
not recommended	10 (18.2)	37 (18.0)		47 (18.1)
Cohort, *n* (%)			0.340	
Arevir	0 (0.0)	6 (2.9)		6 (2.3)
Aquitaine	3 (5.5)	25 (12.2)		28 (10.8)
EuroSIDA	0 (0.0)	0 (0.0)		0 (0.0)
ICONA	11 (20.0)	40 (19.5)		51 (19.6)
KompNet	5 (9.1)	9 (4.4)		14 (5.4)
RFH	12 (21.8)	48 (23.4)		60 (23.1)
Swiss HIV Cohort Study	24 (43.6)	77 (37.6)		101 (38.8)

IAS-USA, IAS-USA HIV-1 drug resistance mutation list (March 2013 update); ABC, abacavir; TDF, tenofovir disoproxil fumarate; 3TC, lamivudine; FTC, emtricitabine; ZDV, zidovudine.

Table 2. Factors associated with virological failure

			ORs of viral rebound >200 RNA copies/mL plasma			
	Cases, $N=76$	Controls, $N=184$	unadjusted OR (95% CI)	P	adjusted[a] OR (95% CI)	P
Gender, *n* (%)						
male	54 (71.1)	153 (83.2)	1.00		1.00	
female	22 (28.9)	31 (16.8)	2.01 (1.07–3.77)	0.029	1.61 (0.73–3.53)	0.239
Ethnicity, *n* (%)						
non-black	69 (90.8)	182 (98.9)	1.00		1.00	
black	7 (9.2)	2 (1.1)	9.23 (1.87–45.53)	0.006	13.62 (2.25–82.49)	0.004
HIV-1 subtype, *n* (%)						
B	52 (68.4)	139 (75.5)	1.00		1.00	
non-B	24 (31.6)	45 (24.5)	1.43 (0.79–2.57)	0.238	1.28 (0.64–2.59)	0.484
Calendar year of starting ART, median (IQR)						
per more recent	2003 (2001–05)	2004 (2002–06)	0.88 (0.79–0.97)	0.010	0.85 (0.72–1.01)	0.072
Time from sample to ART initiation, median (IQR)						
per month longer	2.97 (1.21–5.02)	1.61 (0.00–3.31)	1.04 (1.00–1.08)	0.038	1.07 (1.02–1.12)	0.006
HIV-1 RNA at NNRTI initiation, median (IQR)						
per $\log_{10}$ copies/mL higher	4.85 (4.54–5.30)	4.91 (4.50–5.36)	0.94 (0.58–1.54)	0.817	1.26 (0.68–2.33)	0.466
NNRTI started, *n* (%)						
nevirapine	21 (27.6)	21 (11.4)	1.00		1.00	
efavirenz	55 (72.4)	163 (88.6)	0.34 (0.17–0.66)	0.002	0.43 (0.18–1.02)	0.055
NRTI backbone started, *n* (%)						
recommended: ABC/3TC or TDF/FTC	17 (22.4)	64 (34.8)	1.00		1.00	
alternative: ZDV/3TC or TDF/3TC	36 (47.4)	96 (52.2)	1.41 (0.73–2.73)	0.304	0.84 (0.30–2.32)	0.732
not recommended	23 (30.3)	24 (13.0)	3.61 (1.65–7.89)	0.001	2.39 (0.74–7.69)	0.145
Detection of ≥1 IAS-USA MV prior to ART, any RTI, *n* (%)						
no	52 (68.4)	153 (83.2)	1.00		1.00	
yes	24 (31.6)	31 (16.8)	2.28 (1.23–4.23)	0.009	2.75 (1.35–5.60)	0.005
Detection of ≥1 IAS-USA NRTI MV prior to ART, *n* (%)						
no	68 (89.5)	175 (95.1)	1.00		1.00	
yes	8 (10.5)	9 (4.9)	2.29 (0.85–6.17)	0.102	2.27 (0.76–6.77)	0.140
Detection of ≥1 IAS-USA NNRTI MV prior to ART, *n* (%)						
no	57 (75.0)	158 (85.9)	1.00		1.00	
yes	19 (25.0)	26 (14.1)	2.03 (1.04–3.94)	0.037	2.41 (1.12–5.18)	0.024
Mutational load (RNA copies/mL), *n* (%)						
0	52 (68.4)	153 (83.2)	1.00		1.00	
400–1000	6 (7.9)	8 (4.3)	2.21 (0.73–6.66)	0.160	2.58 (0.68–9.73)	0.162
>1000	18 (23.7)	23 (12.5)	2.30 (1.15–4.60)	0.018	2.81 (1.26–6.24)	0.011

IAS-USA, IAS-USA HIV-1 drug resistance mutation list (March 2013 update); ABC, abacavir; TDF, tenofovir disoproxil fumarate; 3TC, lamivudine; FTC, emtricitabine; ZDV, zidovudine.
[a]Adjusted for calendar year of starting first-line ART, time from sample to ART initiation, viral load at ART initiation, NRTI pair started, NNRTI started, ethnicity, HIV-1 subtype, gender and cohort study.

Subjects' characteristics

Subjects were mostly male, Caucasian and infected through sexual intercourse with mostly HIV-1 subtype B (Table 1). Their median age was 34 years and the median calendar year of ART initiation was 2003. They began ART with a median CD4+ count of 259 cells/mm^3 counts and median HIV-1 RNA levels of 4.9 log copies/mL plasma. Most of them (83.9%) started with efavirenz. The NRTI backbone consisted of drugs currently considered alternative in treatment guidelines (zidovudine/lamivudine or tenofovir/lamivudine) in 50.8% and currently recommended NRTIs (tenofovir disoproxil fumarate/emtricitabine or abacavir/lamivudine) in 31.2%; 18.1% began with NRTI combinations that are no longer recommended (e.g. including didanosine, stavudine, or tenofovir in combination with zidovudine). Baseline

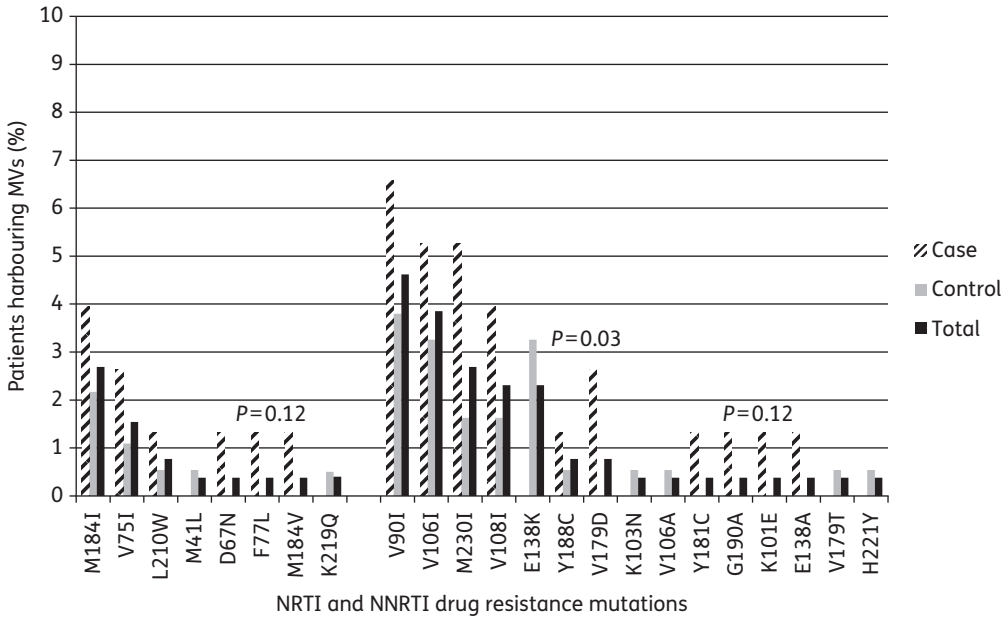

Figure 2. IAS-USA 2013 mutations detected as minority drug resistance mutations by case–control status. Only *P* values <0.2 are reported. Two hundred and forty-five unique samples were included.

characteristics were evenly distributed between subjects with or without MVs with two exceptions: MVs were less frequently observed in women ($P=0.05$), slightly more frequent in subjects receiving currently recommended NRTIs and slightly less frequent in subjects receiving currently alternative NRTIs ($P=0.04$).

MVs

MVs were analysed by next-generation sequencing in plasma samples with a median of 4.91 $\log_{10}$ HIV-1 RNA copies/mL plasma collected 2 (IQR 0–4) months prior to ART initiation (Table 1). Due to amplicon overlap, all relevant amino acid positions in the reverse transcriptase were covered by ≥1000 sequence reads, with median coverage ranging from ≥1500 to 5000 sequence reads (Figure S3). MVs were detected in 55 (21%) subjects: 17 (7%) to NRTIs and 45 (17%) to NNRTIs at frequencies of 1.1%–24.4% (Table S1). Overall, MVs were more frequently observed in cases (24/76; 31.6%) than in controls (31/184; 16.8%) (χ^2 test, $P=0.009$; Table 2). All but three subjects with MVs harboured only one MV. The most frequently observed MVs were M184I (3%), V75I (1.5%) and L210W (1%) for NRTIs and V90I (5%), V106I (4%), M230I (3%), V108I (2%) and E138K (2%) for NNRTIs (Figure 2). The study was not powered to detect significant differences in the prevalence of individual mutations among groups.

Factors associated with virological failure

After adjusting for calendar year of starting first-line ART, time from sample to ART initiation, HIV-1 RNA levels at ART initiation, specific NRTI pair started, NNRTI started, ethnicity, HIV-1 subtype, gender and cohort of enrolment, detection of at least one IAS-USA 2013 MV conferring resistance to RTIs was associated with an increased risk of virological failure to first-line, NNRTI-containing ART [OR=2.75 (95% CI 1.35–5.60), $P=0.005$] (Table 2). Similar associations were observed for detection of at least one IAS-USA 2013 MV resistant to NNRTIs alone [OR=2.41 (95% CI 1.12–5.18), $P=0.024$] or NRTIs alone [OR=2.27 (95% CI 0.76–6.77), $P=0.140$] (Table 2), although the latter did not reach statistical significance.

There was a direct dose–effect relationship between the mutational load of MVs and risk of virological failure. Subjects with 400–1000 MV copies/mL plasma had an intermediate risk [OR=2.58 (95% CI 0.68–9.73), $P=0.162$] whereas those with >1000 MV copies/mL plasma had the highest risk [OR=2.81 (95% CI 1.26–6.24), $P=0.011$].

The magnitude of these associations was consistent in pre-specified groups stratified by baseline NNRTI or NRTI used as well as in the sensitivity analysis including 245 unique samples [OR for at least one IAS-USA RTI MV=2.69 (95% CI 1.30–5.57), $P=0.008$] (Table S2) and excluding sequences containing aligned in-frame stop codons [OR for at least one IAS-USA RTI MV=1.89 (95% CI 0.88–4.04), $P=0.103$] (Table S3).

Other factors associated with increased risk of virological failure included black ethnicity [OR=13.62 (95% CI 2.25–82.49), $P=0.004$] and time from sample to ART initiation [OR=1.07 (95% CI 1.02–1.12) per month longer, $P=0.006$].

Discussion

In this large European case–control study, detection of low-frequency mutations conferring resistance to RTIs was strongly associated with a >2-fold increased risk of virological failure in people initiating first-line ART with two NRTIs plus one NNRTI. This association was consistent regardless of the NRTI backbone started or whether nevirapine or efavirenz was commenced. Moreover, there was a significant dose–effect relationship between the mutational load of MVs and the risk of virological failure.

Similar results were observed in the analyses focusing on the effect associated with the detection of at least one NNRTI or one NRTI mutation, although for the latter the association did not reach statistical significance.

This study confirms and extends previous observations[15,17–21] and to date provides the highest degree of evidence available that low-frequency drug-resistant mutants do impair the efficacy of ART including the NNRTIs nevirapine or efavirenz plus two NRTIs. Interestingly, this was independent from the viral load prior to ART, which was not different in cases and controls. It is also the largest single study available using next-generation sequencing for HIV-1 drug resistance genotyping.

We defined virological failure as a confirmed viraemia rebound of >200 copies/mL after achieving HIV-1 RNA ≤50 copies/mL. This definition excluded subjects who never achieved HIV-1 RNA suppression and might have led to exclusion of people with NNRTI-resistant MVs most strongly associated with drug resistance, such as K103N and Y181C. Indeed, previous studies showed that MVs such as K103N and Y181C strongly predicted early virological failure and lack of HIV-1 RNA suppression.[15,17] Thus, our study design might lead to an underestimation of the impact of MVs. However, we deliberately chose this definition to remove patients with poor adherence, a factor associated with both drug resistance and virological failure, which is difficult to control for in analysis of observational data.

To avoid heterogeneity related to minority drug-resistant HIV-1 variant testing, cDNA synthesis, 454 amplicon generation and 454 pyrosequencing were centralized in two highly experienced European centres, blinded for virological outcomes. Amplicon generation and sequencing is relatively complex with this platform and testing in laboratories with different degrees of expertise could have led to inconsistencies. Parallel analysis of raw 454 data in two independent laboratories blinded for clinical outcomes achieved 100% agreement, ruling out sequence interpretation biases.

The case–control design of this study does not allow a formal estimate of the incidence of virological failure in the cohorts, because the number of cases is fixed at the outset. Nevertheless, we detected MVs more frequently than anticipated (in 21% of subjects overall). In a previous pooled analysis of studies,[15] 14% of subjects infected with WT HIV-1 by population sequencing harboured minority NNRTI-resistant variants. The most prevalent mutations in our population were V90I, V106I and M230I for NNRTIs and M184I, V75I and L210W for NRTIs. Mutations V90I and V106I are polymorphic accessory mutations weakly selected by NNRTIs *in vitro* and *in vivo*. They contribute to reduced susceptibility to etravirine in combination with other mutations, but induce a small, if any, reduction in efavirenz or nevirapine susceptibility even if detected as majority variants.[22] In a recent analysis of 4528 UK patients who commenced efavirenz or nevirapine with at least two NRTIs without major drug resistance mutations, polymorphisms at codons 90 (mostly V90I), 98 (mostly A98S) and 103 (mostly K103R) were each independently associated with increased risk of virological failure.[23] In combination with other mutations, V106I may enhance the level of resistance to efavirenz.[24] The other mutations detected in our study are well known to reduce NNRTI susceptibility. As our study was not powered to discern the effect of individual mutations on the efficacy of first-line, NNRTI-containing ART, it is uncertain whether any of the individual mutations detected exerted a direct effect on NNRTI susceptibility or, in contrast, were surrogate markers of other, perhaps more relevant NNRTI resistance mutations present in the virus population below the detection threshold of 454 pyrosequencing.

In our main analysis, we did not exclude sequences containing stop codons, which may be a marker of poor sequence quality. However, sequence quality or alignment problems were ruled out by visual inspection of alignments. In addition, mutants required a minimum coverage based on the Poisson probability to detect rare events[25] and an even representation in forward and reverse reads to be considered real.[26] Indeed, a sensitivity analysis excluding sequences with stop codons provided similar trends to those observed in the main analysis (Table S3), although trends are non-significant, pointing out a possible role of stop codon-containing sequences in resistance analysis. In fact, the most prevalent mutations in our study correspond to G-to-A mutations and could share a generation mechanism with stop codons, both resulting from APOBEC3G/F editing,[27,28] which is not related to sequence quality. This phenomenon is being further investigated by our group.

As a limitation, we did not collect enough genotypic tests at virological failure to investigate whether the pre-ART MVs were enriched at viral rebound (Table S4). However, such a relationship might not be linear, as the fate of pre-ART variants may also be determined by the specific treatment received and by complex mutational interactions where not only resistance but also fitness and genetic barriers to resistance would play important roles. In previous studies in antiretroviral-naive subjects,[21] NNRTI regimen choice and pre-existing minority NNRTI-resistant variants were both associated with the probability and type of resistance mutations detected after virological failure. Also, our study lacked a formal evaluation of ART adherence given that cohorts do not uniformly collect such data. In previous studies,[19,20] the presence of minority NNRTI resistance mutations and NNRTI adherence were found to be independent predictors of virological failure, but also to modify each other's effects on virological failure. By including people who achieved viral suppression on their initial ART, we should have reduced the inclusion of poorly adherent subjects.

Other factors associated with virological failure in our study were black ethnicity and longer time from sample to ART initiation. Previous studies have linked black ethnicity with virological failure through impaired socioeconomic status and adherence[29] and/or presence of cytochrome P450 polymorphisms associated with a slow metabolizer profile, which lead to increased neurotoxicity by efavirenz.[30,31] The association between longer time from sample to ART initiation, again, might be a marker of poor adherence, overall quality of care or some other unrecognized factors. Indeed, it is conceivable that initiation of ART might be delayed in persons who are perceived to be potentially non-adherent. However, the magnitude of the OR is small and therefore this association is probably not clinically relevant.

In summary, this study confirms and extends previous reports and provides strong evidence that MVs in the reverse transcriptase impair the efficacy of first-line ART including efavirenz or nevirapine. Our findings, together with those previously published, suggest that ultrasensitive HIV-1 genotyping could have a role in improving the outcomes of ART including NNRTIs. Consequently, they also provide a rationale for including ultrasensitive genotyping in HIV-1 resistance surveillance programmes.

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Supplementary data

The Standard Operating Procedure, Figures S1 to S3 and Tables S1 to S4 are available as Supplementary data at *JAC* Online (http://jac.oxfordjournals.org/).

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