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Next-Generation Virus Genotyping for HIV-1 Surveillance and Clinical Management

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Addendum

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Clinical value of ultradeep HIV-1 genotyping and tropism testing in late presenters with advanced disease

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Objective: This article aims to investigate if the detection of preexisting drug-resistant minority variant (DRMV) and/or X4 HIV-1 variants could improve the efficacy of first-line combined antiretroviral therapy (ART) in late presenters.

Design: Post-hoc, combined analysis of two open-label, prospective, randomized clinical trials comparing first-line ART with efavirenz (EFV) vs. ritonavir-boosted protease inhibitor (PI/r)-based regimens in ART-naive, HIV-1-infected patients, with $CD4^+$ T-cell counts less than 100 cells/μl and wild-type HIV-1 by bulk sequencing.

Methods: Pre-ART samples were reanalyzed for the presence of DRMVs and X4 HIV-1 using 454 sequencing. Kaplan–Meier curves and Cox regression were used to evaluate the association between X4 HIV and DRMVs and risk of virological failure.

Results: From 141 evaluable patients, 57 received EFV, and 84 received PI/r, including first-line ART. Median pre-ART $CD4^+$ T-cell counts and HIV-1 RNA levels were 39 cells/μl and 257 424 copies/ml, respectively; 35.5% of patients had X4 HIV variants. Detection of DRMVs leading to an ART-specific cumulative HIVdb score of at least 10 increased the risk of virological failure in patients initiating EFV [log-rank $P = 0.048$, hazard ratio = 4.3 (95% confidence interval: 0.8, 25.0), $P = 0.074$], but not in those starting PI/r. Presence of X4 HIV did not affect virological outcomes, but was associated with impaired $CD4^+$ T-cell count recovery over 2 years (214 vs. 315 cells/μl with X4 vs. R5 HIV-1 tropism, respectively, $P = 0.017$).

Conclusion: Accounting for preexisting DRMVs may improve the outcomes of first-line nonnucleoside reverse transcriptase inhibitor-based ART in late presenters with advanced immune suppression. Presence of X4 HIV-1 at diagnosis predicts impaired immune restoration under ART.

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Keywords: advanced HIV disease, HIV-1 viral tropism, minority drug resistance mutations, ultradeep sequencing, virologic failure

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Introduction

One of the main unsolved public health problems in HIV/AIDS is that, still, more than 30% of people living with HIV in Western countries are diagnosed at late stages of their infection [1], that is, when their $CD4^+$ T-cell counts have already decreased to less than 200 cells/μl or developed an AIDS-defining illness [2]. Late HIV-1 diagnosis is associated with increased mortality and morbidity [1]. Late presenters are more likely to develop antiretroviral therapy (ART) failure [3], suffer AIDS-defining illnesses, transmit HIV to other individuals, and achieve suboptimal immune reconstitution with ART [4]. Up to a third of all HIV-related deaths today are estimated to be consequence of late diagnosis. Late presentation thus implies a huge economic burden to healthcare systems [5–7]. It is crucial to identify and provide late presenters with the best treatment option as early as possible.

Studies in patients with advanced HIV disease have generally compared the efficacy of different ART combinations in virological [8], immunological, and inflammatory [9,10] outcomes, but none has addressed whether HIV-1 tropism or the presence of drug-resistant minority variants (DRMVs), which are not routinely evaluated, could be relevant factors influencing treatment outcomes. In previous studies, first-line efavirenz (EFV)-based ART achieved noninferiority [12] or even superiority [8] in terms of virological efficacy than protease inhibitor-based therapy, being a suitable treatment option for ART-naive patients with advanced disease. However, the efficacy of nonnucleoside reverse transcriptase inhibitors (NNRTIs) may be impaired by the presence of minority NNRTI-resistant variants, which could be missed by current Sanger sequencing genotyping approaches [11]. Minority protease inhibitor-resistant variants have not been shown to affect the efficacy of first-line protease inhibitor therapy [12,13], but studies are scarce, generally underpowered, and often mixed naturally occurring protease polymorphisms with drug-resistant mutations when defining minority variants, thus diluting the effect of resistance mutations having an impact on protease inhibitor treatment outcomes.

HIV-1 tropism might also influence ART outcomes. In a subanalysis of 428 patients of the ArTEN study [14], a prospective, randomized, open-label, noninferiority trial that compared nevirapine vs. atazanavir/ritonavir (ATV/r), each combined with fixed-dose coformulated tenofovir/emtricitabine (TDF/FTC) in antiretroviral-naïve patients with HIV-1 infection, HIV-1 tropism was an independent predictor of virological failure to first-line ART, particularly at week 24 post-ART initiation and in non-B subtypes. It is well known that patients with low $CD4^+$ T-cell counts, like the ones evaluated in this study, are more likely to present CXCR4-using viruses [15]. The ArTEN tropism results, however, have not been confirmed in independent analyses.

Here, we investigated if baseline HIV-1 viral tropism and DRMV testing using next-generation sequencing were associated with virological and immunological outcomes of first-line ART in patients initiating therapy with less than 100 $CD4^+$ T cells/μl in two randomized clinical trials comparing EFV vs. ritonavir-boosted protease inhibitor (PI/r)-based regimens [16].

Methods

Study design

This was a retrospective analysis of baseline clinical samples collected in two prospective, multicentric, open-label, randomized clinical trials comparing the efficacy of first-line ART with EFV vs. indinavir/ritonavir combined with coformulated zidovudine along with lamivudine (Combivir; GlaxoSmithKline) (ADVANZ study [16]), and EFV vs. ATV/r vs. lopinavir/ritonavir, all combined with coformulated TDF/FTC (Truvada; Gilead Sciences Inc.) (ADVANZ-3 study) [16]. Both studies were approved by the ethics committees of the participating hospitals and the Spanish Agency for Medicines and Healthcare Products and conducted in compliance with the Declaration of Helsinki, good clinical practice guidelines, and local regulations. Patients were adequately informed about the study objectives and signed a written informed consent form before enrolment. All patients were ART naive, had $CD4^+$ T-cell counts less than 100 cells/μl, and were infected with wild-type HIV-1 according to bulk sequencing at study entry. Patients were recruited from six Spanish HIV clinical centers between November 2001 and January 2003 (ADVANZ study), and from five centers between September 2007 and February 2010 (ADVANZ-3 study), and were followed during 3 and 2 years, respectively. To homogenize our dataset, we only considered data from the first 2 years of follow-up of both the studies. Virological failure was defined as two consecutive HIV-1 RNA determinations of at least 200 copies/ml or one HIV-1 RNA measurement of at least 1000 copies/ml at or after month 6 of ART. Stored pre-ART samples were reanalyzed for the presence of DRMVs and HIV-1 tropism using 454 sequencing as previously reported (Supplementary methods, http://links.lww.com/QAD/A717) [17,18].

Tropism

$Geno2Pheno_{[454]}$ was used to evaluate tropism directly from raw 454 .sff files. Tropism was defined using the criteria established in the previous MERIT, MOTIVATE-1 and 2, and the A4001029 trials of maraviroc in ART-naïve and experienced patients [18], that is, X4 HIV was defined as the presence of at least 2% of variants with a Geno2Pheno False Positive Rate 3.75% or less. This

definition also includes dual/mixed tropic viruses, as sequencing techniques do not differentiate between them.

HIV-1 resistance

Sequences were analyzed using the Roche/454 proprietary Amplicon Variant Analyzer software (v2.8.0) as described previously [17]. A coverage of at least 500 reads per position was required for further analysis to ensure a minimum opportunity of detecting a low-frequency variant; codons with less than 500 reads were considered 'low covered' and treated as wild type. According to the previous strand-dependent sequencing error patterns and negative control testing results (sequencing of a pNL43 DNA clone), only variants with frequency values on forward and reverse reads within a one log ratio and an overall frequency greater than 0.5% were utilized for downstream analysis. Amplicons with less than a 10% of the expected length were discarded and not considered for further analyses (see [17] for a detailed description of the 454 sequencing and analysis methods, including error controls).

Drug susceptibility was analyzed using Stanford HIVdb (v6.3.1; Stanford University, California, USA). Patients with ART resistance mutations present in at least 20% of their viral population were excluded from downstream analyses. The remaining individuals were classified according to whether mutations detected impacted ART susceptibility or not. Such impact on ART susceptibility was defined as a cumulative ART-specific Stanford HIVdb score ($_{\text{ART}}$HIVdb score) of at least 10 points. For example, a patient with three mutants detected, in whom each mutant was associated with a HIVdb score of 5 to any of the antiretroviral drugs initiated, would be categorized as having an $_{\text{ART}}$HIVdb score of at least 10 ($5+5+5=15$). This procedure ensured that only mutants with some impact on drug susceptibility were considered in the treatment outcomes analysis. It also allowed us to evaluate NNRTI and protease inhibitor-resistant variants together.

Statistical analysis

Baseline patient's characteristics were described overall and according to virological outcome. *P* values were obtained with the χ^2 or Fisher tests for categorical data, and the Mann–Whitney rank sum test for continuous data. Survival analyses including Kaplan–Meier curves and Cox proportional hazards models were used to estimate differences in risk of virological failure by baseline HIV tropism and $_{\text{ART}}$HIVdb score. Separate models were constructed: one taking into account all patients, another one considering only those on EFV, and a third one including patients receiving PI/r therapy exclusively. Separate multivariate Cox proportional hazards models were constructed for all patients, EFV-treated patients, and PI/r-treated patients, using covariates that achieved a *P* value below 0.1 in each corresponding univariate analysis, in addition to the $_{\text{ART}}$HIVdb score. Sensitivity analyses using other definitions, such as presence of at least one IAS-USA 2013 NRTI and/or NNRTI mutation or an $_{\text{ART}}$HIVdb score cutoff of 5, were also performed. Finally, changes in $CD4^+$ T-cell counts through year 2 of follow-up were compared according to $_{\text{ART}}$HIVdb score and HIV tropism, either including all patients or after excluding individuals developing virological failure. Findings were confirmed with a linear mixed models analysis of the slope of $CD4^+$ gains over the study. Statistical analyses and graphs were performed using GraphPad Prism v5.0 (GraphPad Software Inc., La Jolla, California, USA), SigmaPlot v12.5 (SyStat Software Inc., San Jose, California, USA), and R.

Results

Patient's characteristics

Out of 148 patients included in the parental ADVANZ ($n=61$) and ADVANZ-3 ($n=87$) studies, three were excluded from our analysis because of lack of follow-up data. Four additional individuals were excluded because of the existence of primary ART resistance when we considered mutations detected at frequencies of at least 20% of the virus population. This left 141 evaluable patients, who were mostly men (79%), presented with AIDS-defining diseases (54.6%) and had had sex with other men (44%) (Table 1). At the time of ART initiation, the study participants were 39 years old, had 37 $CD4^+$ T cells/μl, and 257 424 HIV-1 RNA copies/ml (all median values); 8.5% were coinfected with the hepatitis B virus and 17.7% with the hepatitis C virus. Fifty-seven (40.4%) patients initiated EFV-based and 84 (59.6%) PI/r–based therapy. There were 29/141 (20.6%) patients who developed virological failure overall, 7/54 (12.3%) in the EFV arm and 22/84 (26.2%) in the PI/r arm.

Virological outcomes by tropism

HIV-1 tropism was evaluable in 139 of 141 (98.6%) patients; in the remaining two (1.4%) individuals, the V3 loop could not be PCR amplified. All patients were infected with subtype B HIV-1. Using the criteria by Swenson *et al.* [18], HIV-1 was R5 in 89 (63.1%) patients and X4 in 50 (35.5%). A similar proportion of X4 HIV was observed among patients developing virological failure or not during follow-up (34.5 vs. 35.7%, respectively, $P=0.755$). There were no differences in time to virological failure by HIV tropism in the Kaplan–Meier analysis (log-rank test = 0.756, Fig. 1a).

Virological outcomes by HIV-1 resistance

Eighteen out of 141 (12.8%) samples did not amplify, leaving 123 (87.2%) evaluable patients. Using 454 sequencing, 19/123 (15.45%) patients had resistance mutations leading to an $_{\text{ART}}$HIVdb score of at least 10 (Table 2). Patients with an $_{\text{ART}}$HIVdb score of at least 10 were more frequent among those developing virological

Table 1. Patient's characteristics at antiretroviral therapy initiation.

Characteristics	Total ($n=141$)	Virological failure ($n=29$)	No virological failure ($n=112$)	P value
Age, median (IQR) (years)	39 (33; 47)	35	40.5	0.065
Gender, *n* (%)				
Men	112 (79%)	21	91	0.429
HIV exposure group, *n* (%)				
MSM	62 (44%)	9 (31%)	53 (47.3%)	0.475
Heterosexual	60 (42.6%)	15 (51.8%)	45 (40.2%)	
IVDU	15 (10.6%)	4 (13.8%)	11 (9.8%)	
Unknown	4 (2.8%)	1 (3.4%)	3 (2.7%)	
RNA HIV-1, median (IQR) (copies/ml)	257 424 (66 400; 500 001)	330 000 (58 159; 671 264)	235 153 (66 393; 500 000)	0.277
$CD4^+$ T-cell counts, median (IQR) (cells/μl)	38.5 (20; 60)	41 (21.5; 61)	37 (19; 60)	0.622
Treatment, *n* (%)				
EFV	57 (40.4%)	7 (24.1%)	50 (44.6%)	0.193
ATV/r	28 (19.9%)	7 (24.1%)	21 (18.8%)	
IDV/r	27 (19.1%)	6 (20.7%)	21 (18.8%)	
LPV/r	29 (20.6%)	9 (31.1%)	20 (17.8%)	
HBV coinfection, *n* (%)				
Positive	12 (8.5%)	2 (6.9%)	10 (8.9%)	0.716
Negative	127 (90.1%)	27 (93.1%)	100 (89.3%)	
Unknown	2 (1.4%)	0	2 (1.8%)	
HCV coinfection, *n* (%)				
Positive	25 (17.7%)	6 (20.7%)	19 (17%)	0.877
Negative	111 (78.8%)	22 (75.9%)	89 (79.5%)	
Unknown	2 (1.4%)	1 (3.4%)	4 (3.5%)	
$_{ART}$HIVdb score				
<10	103 (73%)	20 (69%)	84 (75%)	0.365
≥10	20 (14.2%)	6 (20.7%)	13 (11.6%)	
Nonamplifiable	18 (12.8%)	3 (10.3%)	15 (13.4%)	
Tropism, *n* (%) (≥2% HIV with G2P ≤3.5%) [18]				
CCR5	89 (63.1%)	19 (65.5%)	70 (62.5%)	0.755
CXCR4	50 (35.5%)	10 (34.5%)	40 (35.7%)	
Nonamplifiable	2 (1.4%)	0	2 (1.8%)	
AIDS at baseline				0.888
AIDS	77 (54.6%)	15 (51.8%)	62 (55.4%)	
No AIDS	64 (45.4%)	14 (48.2%)	50 (44.6%)	

ART, antiretroviral therapy; ATV/r, atazanavir/ritonavir; EFV, efavirenz; HBV, hepatitis B virus; HCV, hepatitis C virus; IDV/r, indinavir/ritonavir; IQR, interquartile range; IVDU, intravenous drug user; LPV/r, lopinavir/ritonavir.

failure than in those who did not (20.7 vs. 11.6%, respectively), although such difference was not statistically significant ($P=0.365$). In the Kaplan–Meier analyses, patients with an $_{ART}$HIVdb score of at least 10 achieved virological failure earlier than those with a lower $_{ART}$HIVdb score (Fig. 1b–d). Differences were statistically significant in the analysis of patients initiating EFV (log-rank test $P=0.048$), but not when considering all patients (log-rank test $P=0.116$) or those initiating PI/r (log-rank test $P=0.494$). Similar results were found in sensitivity analyses using other definitions, such as the presence of at least one IAS-USA RTI or NNRTI resistance mutation or an $_{ART}$HIVdb score cutoff of 5 (not shown).

Cox proportional hazards models of risk of virological failure

Overall, baseline factors significantly associated with virological failure in the univariate Cox proportional hazards model (Table 3) were age below 35 years [hazard ratio (HR) = 2.1, 95% confidence interval (CI): 1.0, 4.3, $P=0.051$] and being on the PI/r group relative to EFV [HR = 2.2, 95% CI: 0.9, 5.1, $P=0.072$]. Factors associated with virological failure in patients receiving EFV were having an $_{ART}$HIVdb score of at least 10 (HR = 4.0, 95% CI: 0.86, 20.0, $P=0.077$) and HCV coinfection (HR = 4.6, 95% CI: 0.9, 23.0, $P=0.061$). The only factor associated with virological failure in patients receiving PI/r was age below 35 years (HR = 2.3, 95% CI: 1.0, 5.3, $P=0.051$). In the multivariate Cox proportional hazards analyses, the only factor remaining independently associated with virological failure was having an $_{ART}$HIVdb score of at least 10 (HR = 4.3, 95% CI: 0.8, 25.0, $P=0.074$) in patients initiating EFV. Analyses considering HIV-1 RNA and $CD4^+$ counts as continuous variables showed the same results (not shown).

CD4 evolution by tropism and drug resistance

Overall, median $CD4^+$ T-cell counts increased from 39 (21, 63) cells/μl at baseline to 209 (120, 323) cells/μl at year 1 and 280 (160, 427) cells/μl at year 2. Preexisting

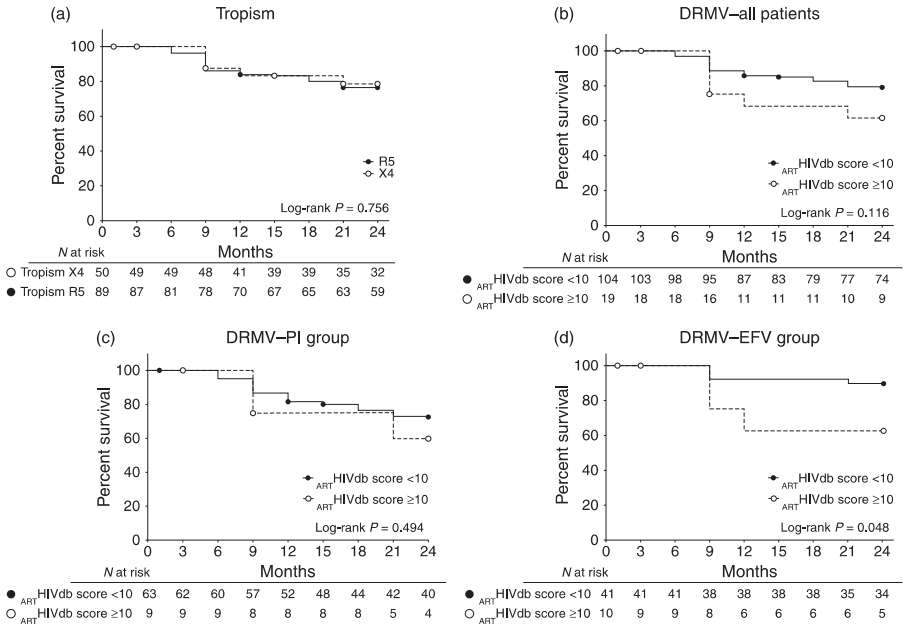

Fig. 1. Risk of virological failure by presence of drug-resistant minority variant or HIV-1 tropism. Kaplan–Meier curves of time to virological failure. Symbols represent censored events. (a) Time to virological failure by HIV-1 tropism considering all patients. (b) Survival analyses by DRMV considering all patients, grouped by $_{ART}$HIVdb score $\geq$10 or <10. (c) Survival analyses by DRMV considering only PI/r-treated patients. (d) Survival analyses by DRMV considering only EFV-treated patients. DRMV, drug-resistant minority variant; EFV, efavirenz; PI/r, ritonavir-boosted protease inhibitor.

drug resistance was not associated with different baseline CD4$^+$ T-cell counts or to different CD4$^+$ T-cell increases during follow-up. In contrast, patients with baseline X4 HIV [18] had significantly lower CD4$^+$ T-cell counts than those infected with an R5 HIV at baseline [30 (14, 51) vs. 43 (26, 66) cells/μl, $P=0.012$], after 1 year of ART [176 (96, 266) vs. 251 (145, 365) cells/μl, $P=0.023$] and after 2 years of ART [214 (127, 316) vs. 315 (176, 461) cells/μl, $P=0.017$] [median (interquartile range) values]. A linear mixed model confirmed the presence of significant differences in CD4$^+$ T-cell slopes after ART initiation between patients with X4 and R5 HIV (7.1 vs. 9.4 cells/μl per month, $P=0.004$) (Fig. 2). Differences between X4 and R5 HIV-1-infected patients remained significant after excluding individuals developing virological failure (data not shown).

Discussion

Choosing the right first-line ART is of critical importance in patients with advanced HIV disease because the short-term risk of severe clinical complications in the event of ART failure is very high [1]. In previous head-to-head comparisons, first-line EFV-based regimens showed equal immunological efficacy [12] but better virological outcomes [16] than PI/r-based regimens in individuals with very low CD4$^+$ T-cell counts. Our study showed, in addition, that preexisting DRMVs increase the risk of virological failure of first-line NNRTI-based ART in this population. Also, although pretreatment HIV-1 tropism did not affect virological outcomes, X4 HIV-1 was associated with impaired immune reconstitution through the first 2 years of ART.

Our study is coherent with the previous reports showing that preexisting DRMVs double the risk of virological failure to first-line ART including NNRTIs [19–21]. In fact, the 2-year rates of virological failure of EFV therapy in patients with no DRMVs were almost negligible. Even with the arrival of new, potent, and well tolerated integrase inhibitors such as dolutegravir, EFV will remain a potent, simple, and affordable treatment alternative for years, more so in resource-limited settings. Our study shows that ultrasensitive genotyping is helpful to identify in which late presenters NNRTI-based ART is unlikely to fail.

Table 2. Patients with mutations associated with an $_{ART}$HIVdb score of at least 10.

Patient ID	HIV-1 RNA (copies/ml)	Treatment arm	Treatment	Mutations found		$_{ART}$HIVdb score
				Mutations impacting ART susceptibility (% in the virus population; sequences per position)	Mutations with no impact in ART susceptibility (% in the virus population; coverage/position)	
1	1 000 000	EFV	EFV, ZDV, 3TC	RT: M184I (1.2; 6713) PR: none	RT: V106I (1.4; 7817); T69S (10.7; 3362); L210M (23.7; 4326). PR: A71V (99.8; 4549); I13V (3.4; 4549); M36I (6.0; 4549); I62V (26.8; 4549); L63P (99.5; 4549); I93L (99.9; 4549)	60
2	406 000	EFV	EFV, ZDV, 3TC	RT: V179D (1.0; 3866); K103R (6.5; 5184) PR: none	RT: none PR: L10I (1.0; 915); V77I (77.3; 915); I93L (85.3; 915); L63P (100; 915)	30
3	330 000	EFV	EFV, ZDV, 3TC	RT: Y181C (0.8, 7328) PR: none	RT: none PR: M36I (3.0; 6533); I64L (94.8; 6533); L63P (100; 6533)	30
4	44 714	EFV	EFV, TDF, FTC	RT: V179D (99.7; 2949); L210W (5.6; 1945) PR: none	RT: T69S (2.5; 2402) PR: V77I (100; 2744)	25
5	261 000	EFV	EFV, ZDV, 3TC	RT: A98G (2.2; 7014); E138Q (36.5; 4378) PR: none	RT: none PR: L10V (6.1; 8139); I64L (99.8; 8139); L63P (100; 8139)	20
6	124 000	EFV	EFV, TDF, FTC	RT: M41L (0.5; 2459) PR: none	RT: T69S (1.3; 2459); V179I (0.8; 3575) PR: N83D (1.6; 4408); D60E (98.6; 4408); M36I (100; 4408)	15
7	1 600 000	EFV	EFV, ZDV, 3TC	RT: V179D (19.9; 3457) PR: none	RT: V106I (0.6; 6413) PR: M36I (7.4; 5561); L10F (99.8; 5561)	10
8	186 762	EFV	EFV, TDF, FTC	RT: V108I (30.3; 5426); K219Q (28.0; 4294) PR: none	RT: K101R (14.9; 7905) PR: A71T (99.1; 6790); M36I (99.2; 6773); I62V (64.3; 6797); L63P (35.5; 6797); I93L (99.9; 6800); D60E (100; 6788)	10
9	88 000	EFV	EFV, TDF, FTC	RT: V179D (22.4; 2256) PR: none	RT: none PR: I50V (0.7; 2527); I13V (99.5; 2522); M36I (5.9; 2522); I64V (99.5; 2525); V77I (85.1; 2525)	10
10	114 911	EFV	EFV, TDF, FTC	RT: E138G (5.9; 2208) PR: none	RT: T69N (2.4; 1444); V179I (99.9; 2806) PR: none	10
11	292 328	PI/r	IDV/r, ZDV, 3TC	RT: D67G (1.0; 2262); T69S (1.2; 2262); T69N (78.8; 2262); T215I (2.4; 5443) PR: none	RT: V106I (73.1; 11325); V179D (15.5; 12089); L210S (0.7; 5443) PR: M36I (92.9; 11643); I62V (0.7; 11643); I64V (24.7; 11643)	55
12	327 800	PI/r	ATV/r, TDF, FTC	RT: none PR: V32I (0.8; 3736); M46I (0.5; 3736)	RT: none PR: V77I (22.3; 3736); L63P (100; 3736)	25
13	371 000	PI/r	IDV/r, ZDV, 3TC	RT: E44D (17.0; 6613); V118I (3.7; 5888) PR: none	RT: none PR: L33I (44.2; 7430); L33V (54.9; 7430); K20R (2.2; 7430); M36I (0.5; 7430); L63P (99.3; 7430); I64L (0.7; 7430); I93L (99.3; 7430)	20
14	151 200	PI/r	ATV/r, TDF, FTC	RT: M41L (6.2; 3668) PR: none	RT: L210M (99.3; 1903); V106I (90.7; 5750) PR: I62V (100; 3630); L63P (100; 3630); I13V (99.8; 3630); M36I (98.6; 3630); V77I (27.4; 3630); M36I (13.3; 3630); L10I (12.2; 3630)	15
15	25 490	PI/r	LPV/r, TDF, FTC	RT: none PR: M46I (0.8; 3559)	RT: V106I (27.0; 5504) PR: G16E (98.9; 3559); I64V (99.8; 3559); V77I (99.8; 4539)	10
16	52 625	PI/r	ATV/r, TDF, FTC	RT: none PR: M46I (12.2; 4674)	RT: V106I (9.8; 6142) PR: L63P (85.5; 4674); I64V (14.3; 4674); V77I (22.8; 4674); V82I (0.5; 4674); I93L (99.4; 4674)	10

17	593 179	PI/r	ATV/r, TDF, FTC	RT: none PR: G73S (1.2; 1867)	RT: A98S (99.2; 6846) PR: I13V (96.5; 1867); G16E (12.0; 1867); I62V (99.6; 1867); L63P (83.7; 1867); I64V (99.8; 1867); I85V (2.5; 1867); D60E (100; 1867); V77I (100; 1867)	10
18	170 000	PI/r	ATV/r, TDF, FTC	RT: none PR: M46I (0.6; 5139)	RT: V106I (8.0; 12700); V75L (2.8; 9564) PR: L33I (0.6; 5139); L33V (99.3; 5139); I62V (33.0; 5151); I64V (100; 5148)	10
19	628 320	PI/r	LPV/r, TDF, FTC	RT: none PR: L33F (7.5; 3781); L33V (6.7; 3781)	RT: L210F (1.9; 2660); E138A (100; 2209) PR: L10V (3.3; 3786); I13V (1.1; 3786); K20M (38.5; 3787); M36I (89.8; 3789); I64V (99.5; 3797)	10

3TC, lamivudine; ART, antiretroviral therapy; ATV/r, atazanavir/ritonavir; EFV, efavirenz; FTC, emtricitabine; IDV/r, indinavir/ritonavir, LPV/r, lopinavir/ritonavir; PI/r, ritonavir-boosted protease inhibitor; PR, protease; RT, retrotranscriptase; TDF, tenofovir; ZDV, zidovudine.

The lack of association of DRMVs with virological outcomes of PI/r-including ART is consistent with the previous studies [12,13,22–24]. Data on the clinical value of DRMVs on first-line PI/r ART is scarce, and definitions of protease inhibitor resistance used in previous studies often included naturally occurring polymorphisms, which have little or no effect on protease inhibitor susceptibility in the absence of major mutations. By using an $_{ART}$HIVdb score, we only considered mutations reducing the virus susceptibility to the ART initiated, including resistance mutations to the NRTI background, and not only protease inhibitor resistance mutations. Even with this approach, however, we were unable to demonstrate an effect of DRMVs on the outcomes of first-line PI/r therapy. Further analyses (not shown) were not able to demonstrate difference in virological outcomes by NRTI backbone (zidovudine/lamivudine vs. TDF/FTC).

HIV-1 tropism did not affect virological outcomes of first-line ART. However, patients with an X4 HIV-1 had lower $CD4^+$ T-cell counts at HIV-1 diagnosis and, more importantly, achieved significantly lower increases in $CD4^+$ T-cell counts through the first 2 years of ART. Our findings contrast with those from the ArTEN trial [14], in which preexisting X4 HIV-1 was independently associated with virological failure to first-line ART including nevirapine or ATV/r, particularly in subtype B viruses, but did not influence $CD4^+$ T-cell count recovery. In a retrospective evaluation of a clinical cohort in London [15], patients with X4 HIV also had lower $CD4^+$ T-cell counts than those with R5 HIV in the absence of ART. However, increases in $CD4^+$ T-cell counts were of similar magnitude once ART was initiated. The lack of association between tropism and virological failure is plausible, as, with the exception of CCR5 and CXCR4 antagonists, antiretroviral drug-mediated HIV-1 inhibition does not depend on HIV-1 tropism.

The impaired immune recovery in patients with X4 HIV-1 is possibly associated with the advanced degree of immune deterioration in our cohort, with median 37 $CD4^+$ T-cell counts/μl at study entry. Thymic $CD34^+$ cells are more easily infected by X4 HIV than R5 HIV, which could explain an increased depletion of thymic production of naive T cells in late presenters with X4 HIV. Although detailed immunological studies are ongoing, from a clinical perspective, our findings indicate that HIV-1 tropism is a marker for impaired immune reconstitution in late presenters and baseline determination of HIV-1 tropism should be included in clinical practice. This important finding should be confirmed in further studies that should also clarify whether X4 HIV-1 could be associated with increased rates of AIDS-defining events or death in late presenters after cART initiation.

To our knowledge, this is the first clinical study evaluating the role of ultrasensitive HIV-1 genotyping in individuals

Table 3. Cox proportional hazard models of risk of virological failure.

		All patients						EFV arm						PI/r- arm					
		Univariate			Multivariate			Univariate			Multivariate			Univariate			Multivariate		
		HR	95% CI	*P* value	HR	95% CI	*P* value	HR	95% CI	*P* value	HR	95% CI	*P* value	HR	95% CI	*P* value	HR	95% CI	*P* value
Age	≥35	1	–	–	1	–	–	1	–	–	–	–	–	1	–	–	1	–	–
	<35	2.1	1; 4.3	0.051	0.6	0.3; 1.4	0.257	0.7	0.1; 5.7	0.734	–	–	–	2.3	1; 5.3	0.051	0.5	0.2; 1.3	0.190
Sex	Male	1	–	–	–	–	–	1	–	–	–	–	–	1	–	–	–	–	–
	Female	1.6	0.7; 3.7	0.229	–	–	–	2.0	0.4; 8.9	0.363	–	–	–	2.1	0.7; 5.5	0.169	–	–	–
Risk group	Heterosexual	1	–	–	–	–	–	1	–	–	–	–	–	1	–	–	–	–	–
	MSM	0.5	0.2; 1.2	0.127	–	–	–	0.5	0.1; 5.8	0.567	–	–	–	0.5	0.2; 1.2	0.112	–	–	–
	IVDU	0.9	0.3; 2.9	0.963	–	–	–	4.2	0.7; 25.5	0.113	–	–	–	0.3	0.1; 2.5	0.291	–	–	–
	Unknown	1.2	0.1; 8.8	0.883	–	–	–	10.2	0.9; 112.2	0.058	–	–	–	–	–	–	–	–	–
HIV-1 RNA (copies/ml)	<100,000	1	–	–	–	–	–	1	–	–	–	–	–	1	–	–	–	–	–
	≥100,000	1.1	0.5; 2.4	0.871	–	–	–	1.3	0.2; 6.5	0.775	–	–	–	1.0	0.4; 2.4	0.998	–	–	–
$CD4^+$ T-cell counts at baseline (cells/μl)[a]	≥50	1	–	–	–	–	–	1	–	–	–	–	–	1	–	–	–	–	–
	<50	1.1	0.5; 2.3	0.869	–	–	–	0.6	0.1; 2.5	0.462	–	–	–	1.3	0.5; 3.1	0.624	–	–	–
Treatment group	EFV	1	–	–	1	–	–	–	–	–	–	–	–	–	–	–	–	–	–
	PI/r	2.2	0.9; 5.1	0.072	1.8	0.7; 4.5	0.189	–	–	–	–	–	–	–	–	–	–	–	–
HCV coinfection	No	1	–	–	–	–	–	1	–	–	1	–	–	1	–	–	–	–	–
	Yes	1.2	0.5; 2.9	0.691	–	–	–	4.6	0.9; 23.0	0.061	3.6	0.7; 18.3	0.111	0.7	0.2; 2.2	0.504	–	–	–
HBV coinfection	No	1	–	–	–	–	–	–	–	–	–	–	–	1	–	–	–	–	–
	Yes	0.7	0.2; 3.0	0.657	–	–	–	–	–	–	–	–	–	0.8	0.2; 3.4	0.752	–	–	–
HIVdb score	<10	1	–	–	1	–	–	1	–	–	1	–	–	1	–	–	1	–	–
	≥10	2.0	0.8; 5.0	0.136	2.0	0.8; 5.0	0.147	4.0	0.8; 20.0	0.077	4.3	0.8; 25.0	0.074	2.0	0.6; 6	0.221	1.8	0.5; 5.2	0.350
Tropism [18]	CCR5	1	–	–	–	–	–	1	–	–	–	–	–	1	–	–	–	–	–
	CXCR4	0.8	0.4; 1.9	0.764	–	–	–	1.5	0.3; 8.6	0.600	–	–	–	0.7	0.2; 1.8	0.493	–	–	–

CI, confidence interval; EFV, efavirenz; HR, hazard ratio; IVDU, intravenous drug user; PI/r, ritonavir-boosted protease inhibitor.
[a]Same results were obtained for continuous variable analyses.

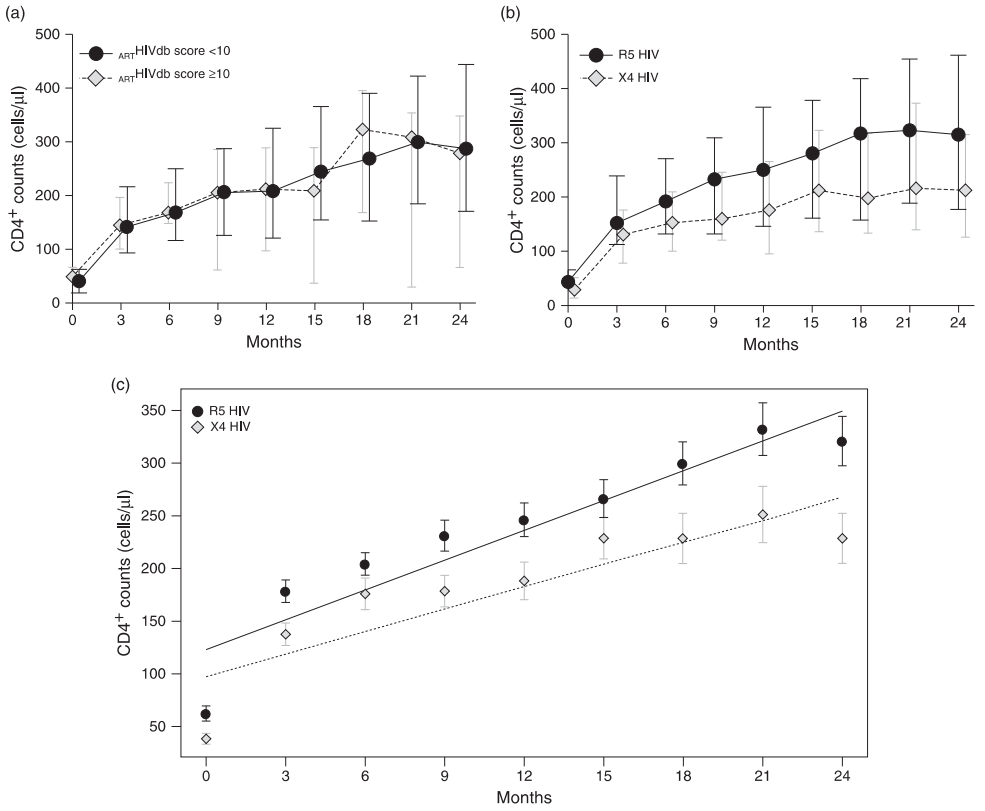

Fig. 2. CD4$^+$ T-cell evolution by baseline drug-resistant minority variant or HIV-1 tropism. (a) Median (interquartile range) CD4$^+$ T-cell counts by DRMV considering all patients, grouped by $_{ART}$HIVdb score ≥10 or <10. (b) Median (interquartile range) CD4$^+$ T-cell counts by HIV-1 tropism (*$P = 0.017$ at month 24). (c) CD4$^+$ T-cell slopes after ART initiation by HIV-1 tropism ($P = 0.004$). ART, antiretroviral therapy; DRMV, drug-resistant minority variant.

with advanced HIV disease. Some strengths of the study include the prospective, randomized nature of the parent trials, which minimizes selection biases and allows a comprehensive and high-quality monitoring of clinical and laboratory data; the high median HIV-1 RNA levels at the time of genotyping, which ensures adequate sampling of the viral population to detect minority variants with high sensitivity; the prolonged prospective follow-up, which allows capturing events up to 2 years; the very low CD4$^+$ T-cell counts at study entry, which allows studying the impact of ultrasensitive genotyping in a population with the greatest need of accurate first-line ART prescription; and the simultaneous evaluation of HIV-1 resistance and tropism on virological outcomes of first-line ART.

A number of limitations are also evident. The main weakness of the study is its small sample size, particularly when EFV and PI/r arms are analyzed separately, which limits the statistical power to detect differences. Also, with the exception of ATV/r, two protease inhibitors (indinavir/ritonavir and lopinavir/ritonavir) used in this study are no longer recommended for first-line ART. However, the principle of investigating the role of DRMVs in patients receiving PI/r remains valid in this setting. Technical limitations related to the read length and the presence of PCR-derived recombination limit our ability to establish true mutational linkage, which could be useful particularly to assess protease inhibitor resistance. Formal adherence or ethnicity information data were not available to this analysis. Previous studies in ART-naive patients showed that both the presence of DRMVs and suboptimal adherence were independent risk factors for virological failure to first-line NNRTI ART but also potentiated each other's effects on virologic failure [25]. Also, the effect of DRMVs was found to be

independent of the ethnicity in previous studies [21]. The current study was performed in a Spanish population almost exclusively conformed by whites infected with a subtype B virus, so it is unlikely that ethnicity could confound our findings. We cannot rule out that our sequencing accuracy, sensitivity, and reproducibility might differ in other subtypes. Study participants had been infected and without treatment for a long time, and it is possible that they have had lost detectability of minority drug-resistant variants, even with ultradeep-sequencing techniques. These were post-hoc analyses not planned initially which, by definition, might conform to neither the population nor the randomization model of statistical inference. In our case, however, the study endpoints were clearly defined and consistent with the overall study design.

Previous studies have shown that ultrasensitive HIV-1 genotyping using modern next-generation sequencing can provide clinical benefits to ART-naïve [19,20] and experienced [17] HIV-1-infected patients by improving the sensitivity of drug resistance and tropism detection over Sanger sequencing, while maintaining high diagnostic robustness. Our study shows that the efficacy of first-line ART in late presenters can be improved by using ultrasensitive HIV-1 genotyping to evaluate the presence of drug-resistant HIV-1 before treatment initiation. Detection of X4 HIV-1, in contrast, identifies patients more likely to have impaired immune reconstitution under first-line ART, who might benefit of additional immune-boosting approaches. Results from this study likely apply to other next-generation platforms, which have demonstrated technical equivalence to 454 sequencing [26] and are already providing advantages in throughput, workload, and cost. Although more data are needed, ultrasensitive HIV-1 genotyping might be a valuable tool in the clinical management of late presenters with advanced disease.

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Role of the authors: M.C. has participated in conceiving and designing the study, performing the laboratory work, collecting the data, analyzing and interpreting the data, writing the manuscript, and approving the final version of the manuscript. C.M. has participated in conceiving and designing the study, collecting the data, analyzing and interpreting the data, writing the manuscript, and approving the final version of the manuscript. M.N.J. has contributed to the analysis and interpretation of the data, providing critical revisions that are important for the intellectual content, and final approval of the version to be published. E.F. and P.D. have participated in conceiving and designing the study, providing critical revisions that are important for the intellectual content, and approving the final version of the manuscript. S.P.Á. has participated in statistical analyses and interpreting the data, revising the

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Conflicts of interest

The authors have read the journal's policy and have the following conflicts. D.P. has received honoraria and speakers' fees from Abbott, Bristol-Myers Squibb, Boehringer Ingelheim, Gilead, GlaxoSmithKline, Janssen-Cilag, Merck, Pfizer, Janssen, and ViiV Healthcare. P.D. has received advisory, consultant, and/or lecture fees and has been a data safety monitoring board member for Abbott, Boehringer-Ingelheim, Bristol-Myers Squibb, Gilead Sciences, Janssen, Merck Sharp & Dohme, ViiV, and Ferrer. B.C. has been a consultant on advisory boards or participated in speakers' bureaus or conducted clinical trials with Boehringer-Ingelheim, Abbott, GlaxoSmithKline, Gilead, Janssen, Merck, Shionogi, and ViiV. J.M.G. has received research funding, consultancy fees, or lecture sponsorship, or served on advisory boards, for Abbott, Boehringer-Ingelheim, Bristol-Myers Squibb, Gilead Sciences, Janssen, and Merck Sharp & Dohme. J.M.M. has received research funding, consultancy fees, or lecture sponsorship, or served on advisory boards, with Cubist Pharmaceuticals, Novartis, Abbott Laboratories, Bristol-Myers Squibb, Gilead Sciences, Merck, Pfizer, Theravance, Boehringer-Ingelheim, GlaxoSmithKline, Janssen-Cilag, Dohme, Roche, Schering-Plough, and ViiV Healthcare. R.P. has received consulting fees from Pfizer and grant support from Pfizer, Roche Diagnostics, Siemens, Merck, and Boehringer-Ingelheim. All other authors report no potential conflicts.

References

1. Mocroft A, Lundgren JD, Sabin ML, Monforte Ad, Brockmeyer N, Casabona J, *et al*. **Risk factors and outcomes for late presentation for HIV-positive persons in Europe: results from the Collaboration of Observational HIV Epidemiological Research Europe Study (COHERE).** *PLoS Med* 2013; **10**:e1001510.
2. Antinori A, Coenen T, Costagiola D, Dedes N, Ellefson M, Gatell J, *et al*. **Late presentation of HIV infection: a consensus definition.** *HIV Med* 2011; **12**:61–64.
3. Battegay M, Fluckiger U, Hirschel B, Furrer H. **Late presentation of HIV-infected individuals.** *Antivir Ther* 2007; **12**:841–851.
4. Robbins GK, Spritzler JG, Chan ES, Asmuth DM, Gandhi RT, Rodriguez BA, *et al*. **Incomplete reconstitution of T cell subsets on combination antiretroviral therapy in the AIDS Clinical Trials Group protocol 384.** *Clin Infect Dis* 2009; **48**:350–361.
5. Fleishman JA, Yehia BR, Moore RD, Gebo KA. **The economic burden of late entry into medical care for patients with HIV infection.** *Med Care* 2010; **48**:1071–1079.
6. Krentz HB, Gill J. **Despite CD4 cell count rebound the higher initial costs of medical care for HIV-infected patients persist 5 years after presentation with CD4 cell counts less than 350 μl.** *AIDS* 2010; **24**:2750–2753.
7. Krentz HB, Gill MJ. **The direct medical costs of late presentation (<350/mm) of HIV infection over a 15-year period.** *AIDS Res Treat* 2012; **2012**:757135.
8. Sierra-Madero J, Villasis-Keever A, Méndez P, Mosqueda-Gómez JL, Torres-Escobar I, Gutiérrez-Escolano F, *et al*. **Prospective, randomized, open label trial of efavirenz vs lopinavir/ritonavir in HIV+ treatment-naive subjects with CD4+ <200 cell/mm^3 in Mexico.** *J Acquir Immune Defic Syndr* 2010; **53**:582–588.
9. Miró J, Manzardo C, Ferrer E, Loncà M, Guardo AC, Podzamczer D, *et al*. **Immune reconstitution in severely immunosuppressed antiretroviral-naïve HIV-1-infected patients taking antiretroviral regimens based on efavirenz, lopinavir-ritonavir, and atazanavir-ritonavir: 48-week results of a randomized controlled trial (the Advanz)**. In: *6th IAS Conference on HIV Pathogenesis and Treatment; 2011*; Rome, Italy.
10. Miro J, Manzardo C, Guardo C, Lozano F, Ferrer E, Domingo P, *et al*. **Effect of combined antiretroviral therapy (cART) on bacterial translocation, inflammation, coagulation and immune activation in advanced (<100 CD4+ cells/mm^3) antiretroviral-naïve patients at week 48**. In: *19th Conference on Retroviruses and Opportunistic Infections; 2012*; Seattle, Washington.
11. Kuritzkes DR, Lalama CM, Ribaudo HJ, Marcial M, Meyer WA, Shikuma C, *et al*. **Preexisting resistance to nonnucleoside reverse-transcriptase inhibitors predicts virologic failure of an efavirenz-based regimen in treatment-naive HIV-1-infected subjects.** *J Infect Dis* 2008; **197**:867–870.
12. Lataillade M, Chiarella J, Yang R, DeGrosky M, Uy J, Seekins D, *et al*. **Virologic failures on initial boosted-PI regimen infrequently possess low-level variants with major PI resistance mutations by ultra-deep sequencing.** *PLoS One* 2012; **7**:e30118.
13. Lataillade M, Chiarella J, Yang R, Schnittman S, Wirtz V, Uy J, *et al*. **Prevalence and clinical significance of HIV drug resistance mutations by ultra-deep sequencing in antiretroviral-naïve subjects in the CASTLE study.** *PLoS One* 2010; **5**:e10952.
14. Seclén E, Soriano V, González MM, Martín-Carbonero L, Gellermann H, Distel M, *et al*. **Impact of baseline HIV-1 tropism on viral response and CD4 cell count gains in HIV-infected patients receiving first-line antiretroviral therapy.** *J Infect Dis* 2011; **204**:139–144.
15. Waters L, Mandalia S, Randell P, Wildfire A, Gazzard B, Moyle G. **The impact of HIV tropism on decreases in CD4 cell count, clinical progression, and subsequent response to a first antiretroviral therapy regimen.** *Clin Infect Dis* 2008; **46**:1617–1623.
16. Miró JM, Manzardo C, Pich J, Domingo P, Ferrer E, Arribas JR, *et al*. **Immune reconstitution in severely immunosuppressed antiretroviral-naive HIV type 1-infected patients using a nonnucleoside reverse transcriptase inhibitor-based or a boosted protease inhibitor-based antiretroviral regimen: three-year results (The Advanz Trial): a randomized, controlled trial.** *AIDS Res Hum Retroviruses* 2010; **26**:747–757.

17. Pou C, Noguera-Julian M, Pérez-Álvarez S, García F, Delgado R, Dalmau D, *et al*. **Improved prediction of salvage antiretroviral therapy outcomes using ultrasensitive HIV-1 drug resistance testing.** *Clin Infect Dis* 2014; **59**:578–588.
18. Swenson LC, Mo T, Dong WWY, Zhong X, Woods CK, Thielen A, *et al*. **Deep V3 sequencing for HIV type 1 tropism in treatment-naive patients: a reanalysis of the MERIT trial of maraviroc.** *Clin Infect Dis* 2011; **53**:732–742.
19. Paredes R, Lalama CM, Ribaudo HJ, Schackman BR, Shikuma C, Giguel F, *et al*. **Preexisting minority drug-resistant HIV-1 variants, adherence, and risk of antiretroviral treatment failure.** *J Infect Dis* 2010; **201**:662–671.
20. Li JZ, Paredes R, Ribaudo HJ, Kozal MJ, Svarovskaia ES, Johnson JA, *et al*. **Impact of minority nonnucleoside reverse transcriptase inhibitor resistance mutations on resistance genotype after virologic failure.** *J Infect Dis* 2013; **207**:893–897.
21. Li JZ, Paredes R, Ribaudo HJ, Svarovskaia ES, Metzner KJ, Kozal MJ, *et al*. **Low-frequency HIV-1 drug resistance mutations and risk of NNRTI-based antiretroviral treatment failure: a systematic review and pooled analysis.** *JAMA* 2011; **305**:1327–1335.
22. Metzner KJ, Rauch P, von Wyl V, Leemann C, Grube C, Kuster H, *et al*. **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.** *J Infect Dis* 2010; **201**:1063–1071.
23. Simen BB, Simons JF, Hullsiek KH, Novak RM, Macarthur RD, Baxter JD, *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.
24. Peuchant O, Thiébaut R, Capdepont S, Lavignolle-Aurillac V, Neau D, Morlat P, *et al*. **Transmission of HIV-1 minority-resistant variants and response to first-line antiretroviral therapy.** *AIDS* 2008; **22**:1417–1423.
25. Li JZ, Paredes R, Ribaudo H, Svarovskaia ES, Kozal MJ, Hullsiek KH, *et al*. **Relationship between minority NNRTI resistance mutations, adherence, and the risk of virologic failure.** *AIDS* 2012; **26**:185–192.
26. Archer J, Weber J, Henry K, Winner D, Gibson R, Lee L, *et al*. **Use of four next-generation sequencing platforms to determine HIV-1 coreceptor tropism.** *PLoS One* 2012; **7**:e49602.

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Primary resistance to integrase strand-transfer inhibitors in Europe

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Objectives: The objective of this study was to define the natural genotypic variation of the HIV-1 integrase gene across Europe for epidemiological surveillance of integrase strand-transfer inhibitor (InSTI) resistance.

Methods: This was a multicentre, cross-sectional study within the European SPREAD HIV resistance surveillance programme. A representative set of 300 samples was selected from 1950 naive HIV-positive subjects newly diagnosed in 2006–07. The prevalence of InSTI resistance was evaluated using quality-controlled baseline population sequencing of integrase. Signature raltegravir, elvitegravir and dolutegravir resistance mutations were defined according to the IAS-USA 2014 list. In addition, all integrase substitutions relative to HXB2 were identified, including those with a Stanford HIVdb score ≥10 to at least one InSTI. To rule out circulation of minority InSTI-resistant HIV, 65 samples were selected for 454 integrase sequencing.

Results: For the population sequencing analysis, 278 samples were retrieved and successfully analysed. No signature resistance mutations to any of the InSTIs were detected. Eleven (4%) subjects had mutations at resistance-associated positions with an HIVdb score ≥10. Of the 56 samples successfully analysed with 454 sequencing, no InSTI signature mutations were detected, whereas integrase substitutions with an HIVdb score ≥10 were found in 8 (14.3%) individuals.

Conclusions: No signature InSTI-resistant variants were circulating in Europe before the introduction of InSTIs. However, polymorphisms contributing to InSTI resistance were not rare. As InSTI use becomes more widespread, continuous surveillance of primary InSTI resistance is warranted. These data will be key to modelling the kinetics of InSTI resistance transmission in Europe in the coming years.

Introduction

HIV integrase is a key enzyme for retroviral replication and one of the main targets of modern HIV therapy.[1,2] Integrase strand-transfer inhibitors (InSTIs) reached clinical practice in Europe in 2007–08, after proving their efficacy in antiretroviral treatment-naive and -experienced subjects.[3–6] Virological failure (VF) to the first-generation InSTIs raltegravir and elvitegravir is associated with

development of resistance through three mutually exclusive pathways characterized by one signature resistance mutation in the catalytic domain of the enzyme, i.e. Y143R/C, N155H or Q148K/R/H, alongside accessory mutations that improve viral fitness or further reduce InSTI susceptibility.[7–10] The resistance profile of the second-generation InSTI dolutegravir is being defined, as few subjects developed VF in clinical trials and data from routine care are still scarce. Viruses with Q148K/R/H plus at least one additional mutation, however, may also affect susceptibility to dolutegravir.[11,12] Based on the low genetic barrier of first-generation InSTIs and with the increasing use of them in clinical practice, surveillance of transmitted InSTI-resistant HIV will be a key to optimizing InSTI efficacy. Primary InSTI resistance is still rare. However, it has started to be reported[13,14] and will likely increase in the coming years. In addition, up to 34% of published sequences, 56% of those obtained from recent HIV infection,[15] contain polymorphisms in the integrase,[16] which modulate InSTI resistance in particular to raltegravir and elvitegravir and are frequently observed in InSTI VF. In this study, we performed a systematic, representative description of the natural sequence variation of the integrase gene across Europe, before InSTI drugs were commercially available. We also aimed to clarify the chances that spontaneously generated InSTI-resistant mutants could be circulating as minority species and be missed by routine population sequencing approaches.

Methods

A sample of 300 subjects was randomly selected from 1950 individuals enrolled in the European SPREAD programme in 2006–07, before InSTIs were introduced into routine clinical care in Europe. SPREAD is a prospective HIV-1 resistance surveillance programme that collects representative data on the spread of HIV-1 resistance among newly diagnosed patients from all risk groups in Europe. Ethics requirements were fulfilled according to the procedure described in the ethics committee contract. Additionally, written informed consent was obtained for each patient. Population sequencing of plasma HIV-1 was performed (ViroSeq® HIV-1 Genotyping System, Abbott; Trugene® HIV-1 Genotyping Kit, Siemens; or in-house methods) in laboratories that successfully participated in the SPREAD quality control (QC) programme for population sequencing. Samples from laboratories that did not participate in the integrase sequencing QC programme or did not successfully meet the QC criteria were tested by one of the qualified laboratories that passed the QC programme within the SPREAD network using the above-mentioned commercial or in-house Sanger sequencing methods. HIV subtypes were determined using Rega Subtyping Tool v2 based on *pol* sequence data.[17] To screen for circulating low-frequency InSTI-resistant mutants, ultradeep integrase sequencing was additionally attempted in 65 subjects randomly selected from those included in the population sequencing analyses, using a 454 FLX Genome Sequencer with Titanium chemistry and a 1% threshold for mutant detection. Sequences were analysed using the Roches's proprietary Amplicon Variant Analyser software (v2.7). Sample contamination was ruled out by similarity analysis both against a pNL4.3 reference sequence and by per-amplicon phylogenetic analysis of all sequences >1% within an ultradeep sequencing run. Given the lack of a widely accepted list of integrase mutations for surveillance, we first listed all substitutions relative to the reference sequence HXB2 (GenBank accession number K03455) according to their frequency in the patient population. We then evaluated which substitutions achieved an HIVdb score ≥10 to at least one InSTI, representing substitutions with potential impact on InSTI susceptibility. We also listed integrase mutations included in the IAS-USA list (July 2014 update). 'Signature mutations' were: Y143R/C/H, N155H and Q148K/R/H for raltegravir; T66I, E92Q, F121Y, S147G, Q148R and N155H for elvitegravir; and G140S and Q148H for dolutegravir.

Results

Samples were retrieved and successfully analysed by population sequencing in 278 out of the 300 (92.7%) subjects selected. The prevalence of transmitted drug resistance mutations found by Sanger sequencing for PIs, NRTIs and NNRTIs was 2.5%, 9.71% and 7.91%, respectively. No signature InSTI mutations were detected. By contrast, we observed integrase-associated mutations with an HIVdb score ≥10 in 11 (4.0%) patients (Table 1 and Table S1, available as Supplementary data at *JAC* Online). Samples unsuccessfully processed had a median (IQR) number of copies/mL of 57 000 (13 212–374 154). The HIV subtype from

Table 1. Subject characteristics and summary of sequencing results

All subjects, *n* (%)	278 (100)
Male, *n* (%)	231 (83.0)
Continent of origin, *n* (%)	
western Europe	180 (64.7)
eastern Europe	48 (17.3)
sub-Saharan Africa	20 (7.2)
Latin America	16 (5.8)
others	14 (5.0)
CDC class, *n* (%)	
A	230 (82.7)
B	21 (7.5)
C	22 (8.0)
unknown	5 (1.8)
CD4+ T count (cells/μL), median	411
Route of transmission, *n* (%)	
MSM/bisexual	180 (64.8)
heterosexual	61 (21.9)
IVDU	5 (1.8)
other	32 (11.5)
Viral subtype, *n* (%)	
B	186 (67.0)
C	15 (5.4)
A	11 (4.0)
F	12 (4.3)
G	6 (2.1)
D	1 (0.3)
unknown	47 (16.9)
Summary of Sanger sequencing, *n* (%)	
IAS-USA integrase mutations	5 (1.8) [74M (2), 97A (2) and 138A]
HIVdb score ≥10	11 (4.0)
Summary of 454 sequencing (*n*=56 subjects), *n* (%)	
IAS-USA integrase mutations	0
HIVdb score ≥10	8 (14.3)

these samples was mainly subtype B ($n=12$; 54.55%) and subtype G ($n=5$; 22.72%).

454 data were obtained from 56/65 (86.1%) subjects. Most of them (85.2%) were infected with subtype B HIV-1. Fifty of them (89.3%) had WT protease, reverse transcriptase and integrase by Sanger sequencing, whereas 6 (10.7%) had transmitted resistance to at least two antiretroviral drug classes. The median (IQR) coverage was 4593 (3066–6598) reads per substitution found. Again, no InSTI signature mutations were detected. However, 8/56 subjects (14.3%) had integrase substitutions with an HIVdb score ≥10 (Table S2). Of these, mutation E157Q was found in five (8.9%) individuals, in two of them as a low-frequency variant. The following mutations were found in one subject each: H51Y, G163R, both as low-frequency variants (1.9; 2.6% in the virus population each); G163K as major variant (100% in the virus population); E157Q was detected at a 22.1% frequency in a subject with transmitted Q58E mutations in the protease and D67N and K219Q mutations in the reverse transcriptase. No integrase substitutions with an HIVdb score ≥10 were detected in the remaining five subjects with transmitted dual-class resistance.

Discussion

No signature InSTI resistance mutations were circulating in Europe before InSTI introduction, although potentially relevant polymorphisms could be observed. This study also indicated a limited utility of ultrasensitive genotyping for surveillance of InSTI-resistant minority variants at present, which might change if the burden of transmitted InSTI resistance increases. Elvitegravir and raltegravir have a low genetic barrier to resistance and extensive overlap in their drug resistance profiles.[18] Raltegravir is often prescribed as salvage therapy to subjects with MDR HIV who could select for InSTI resistance, which could be transmitted to newly infected subjects. Dolutegravir has a higher genetic barrier to resistance than elvitegravir and raltegravir, but its long-term potency might be reduced in the presence of Q148R/H/K plus one or two additional mutations. As dolutegravir is also often prescribed as salvage ART, dolutegravir resistance might also evolve in European populations in the coming years. Continued surveillance of InSTI resistance in Europe is thus warranted, including periodic re-evaluations of the usefulness of ultrasensitive genotyping technologies, which nowadays allow faster monitoring of transmitted resistance, particularly with large sample sets.

Substitutions detected with at least some presumed impact on ART susceptibility (i.e. having an HIVdb score ≥10) were E157Q, G163R/K, L74M, T97A, E138A, S153F and R263K. E157Q is a polymorphic accessory mutation weakly selected in patients receiving raltegravir and selected *in vitro* by elvitegravir. G163R/K are non-polymorphic mutations in all subtypes except F, often selected in patients receiving raltegravir. However, their effect on InSTIs has not yet been well studied. L74M is a polymorphic accessory mutation selected in patients receiving raltegravir, elvitegravir and dolutegravir, which does not reduce InSTI susceptibility unless it is found in combination with other InSTI resistance mutations. T97A is a polymorphic accessory mutation selected by raltegravir and elvitegravir that occurs in 1%–5% of viruses from untreated persons. Combined with Y143C/R, it markedly reduces raltegravir susceptibility, although it has minimal effect alone. E138A is a non-polymorphic accessory resistance mutation usually occurring in combination with Q148 mutations, selected in patients receiving raltegravir, elvitegravir and dolutegravir. It is associated with >100-fold reduction in raltegravir and elvitegravir susceptibility and up to 10-fold reduced dolutegravir susceptibility in combination with Q148. S153F is selected *in vitro* by dolutegravir and is a rare non-polymorphic mutation, reducing raltegravir and dolutegravir susceptibility by 2-fold and elvitegravir susceptibility by 4-fold. R263K is a non-polymorphic mutation selected in patients receiving raltegravir and dolutegravir and *in vitro* by elvitegravir and dolutegravir, reducing raltegravir, dolutegravir and elvitegravir susceptibility by ∼2-fold, 2-fold and 3- to 5-fold, respectively.[19,20]

Therefore, as long as transmitted InSTI resistance remains at negligible levels, there is no clinical need to perform integrase genotyping before initiating InSTI therapy. However, continued surveillance is key to informing clinicians and policymakers about when baseline genotyping should be systematically recommended. It is essential to perform integrase gene genotyping in subjects failing InSTI therapy, as new InSTIs with alternative resistance profiles are under development and subjects should not be kept on failing InSTI regimens.

In conclusion, no signature InSTI-resistant variants were circulating in Europe before introducing InSTIs. However, polymorphisms that could contribute to InSTI resistance were not rare. As InSTI use becomes more widespread, continuous surveillance of primary InSTI resistance is warranted. This study provides an extensive assessment of primary InSTI resistance based on a representative sample of the European epidemic and is a robust baseline comparator for future InSTI surveillance, which will be key to modelling the kinetics of InSTI resistance patterns of transmission in Europe in the coming years.

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Transparency declarations

None to declare.

Supplementary data

Tables S1 and S2 are available as Supplementary data at *JAC* Online (http://jac.oxfordjournals.org/).

References

1 Marrazzo JM, del Rio C, Holtgrave DR *et al*. HIV prevention in clinical care settings: 2014 recommendations of the International Antiviral Society-USA Panel. *JAMA* 2014; **312**: 390–409.

2 Department of Health and Human Services Panel on Antiretroviral Guidelines for Adults and Adolescents. *Guidelines for the Use of Antiretroviral Agents in HIV-1-Infected Adults and Adolescents*. http://aidsinfo.nih.gov/contentfiles/lvguidelines/AdultandAdolescentGL.pdf.

3 Correll T, Klibanov OM. Integrase inhibitors: a new treatment option for patients with human immunodeficiency virus infection. *Pharmacotherapy* 2008; **28**: 90–101.

4 Hughes A, Barber T, Nelson M. New treatment options for HIV salvage patients: an overview of second generation PIs, NNRTIs, integrase inhibitors and CCR5 antagonists. *J Infect* 2008; **57**: 1–10.

5 Eron JJ, Rockstroh JK, Reynes J *et al*. Raltegravir once daily or twice daily in previously untreated patients with HIV-1: a randomised, active-controlled, phase 3 non-inferiority trial. *Lancet Infect Dis* 2011; **11**: 907–15.

6 Steigbigel RT, Cooper DA, Kumar PN *et al*. Raltegravir with optimized background therapy for resistant HIV-1 infection. *N Engl J Med* 2008; **359**: 339–54.

7 Cooper DA, Steigbigel RT, Gatell JM *et al*. Subgroup and resistance analyses of raltegravir for resistant HIV-1 infection. *N Engl J Med* 2008; **359**: 355–65.

8 Goethals O, Clayton R, Van Ginderen M *et al*. Resistance mutations in human immunodeficiency virus type 1 integrase selected with elvitegravir confer reduced susceptibility to a wide range of integrase inhibitors. *J Virol* 2008; **82**: 10366–74.

9 Malet I, Delelis O, Valantin M-A *et al*. Mutations associated with failure of raltegravir treatment affect integrase sensitivity to the inhibitor in vitro. *Antimicrob Agents Chemother* 2008; **52**: 1351–8.

10 Loizidou EZ, Kousiappa I, Zeinalipour-Yazdi CD *et al*. Implications of HIV-1 M group polymorphisms on integrase inhibitor efficacy and resistance: genetic and structural in silico analyses. *Biochemistry* 2009; **48**: 4–6.

11 Castagna A, Maggiolo F, Penco G *et al*. Dolutegravir in antiretroviral-experienced patients with raltegravir- and/or elvitegravir-resistant HIV-1: 24-week results of the phase III VIKING-3 study. *J Infect Dis* 2014; **210**: 354–62.

12 Underwood MR, Johns BA, Sato A *et al*. The activity of the integrase inhibitor dolutegravir against HIV-1 variants isolated from raltegravir-treated adults. *J Acquir Immune Defic Syndr* 2012; **61**: 297–301.

13 Young B, Fransen S, Greenberg KS *et al*. Transmission of integrase strand-transfer inhibitor multidrug-resistant HIV-1: case report and response to raltegravir-containing antiretroviral therapy. *Antivir Ther* 2011; **16**: 253–6.

14 Boyd SD, Maldarelli F, Sereti I *et al*. Transmitted raltegravir resistance in an HIV-1 CRF_AG-infected patient. *Antivir Ther* 2011; **16**: 257–61.

15 Low A, Prada N, Topper M *et al*. Natural polymorphisms of human immunodeficiency virus type 1 integrase and inherent susceptibilities to a panel of integrase inhibitors. *Antimicrob Agents Chemother* 2009; **53**: 4275–82.

16 Rhee S-Y, Liu TF, Kiuchi M *et al*. Natural variation of HIV-1 group M integrase: implications for a new class of antiretroviral inhibitors. *Retrovirology* 2008; **5**: 74.

17 Alcantara LCJ, Cassol S, Libin P *et al*. A standardized framework for accurate, high-throughput genotyping of recombinant and non-recombinant viral sequences. *Nucleic Acids Res* 2009; **37**: W634–42.

18 Marinello J, Marchand C, Mott BT *et al*. Comparison of raltegravir and elvitegravir on HIV-1 integrase catalytic reactions and on a series of drug-resistant integrase mutants. *Biochemistry* 2008; **47**: 9345–54.

19 Shafer RW. Rationale and uses of a public HIV drug-resistance database. *J Infect Dis* 2006; **194** Suppl 1: S51–8.

20 Liu TF, Shafer RW. Web resources for HIV type 1 genotypic-resistance test interpretation. *Clin Infect Dis* 2006; **42**: 1608–18.

Research Letter

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Treatment options after virological failure of first-line tenofovir-based regimens in South Africa: an analysis by deep sequencing

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In a South African cohort of participants living with HIV developing virological failure on first-line tenofovir disoproxyl fumarate (TDF)-based regimens, at least 70% of participants demonstrated TDF resistance according to combined Sanger and MiSeq genotyping. Sanger sequencing missed the K65R mutation in 30% of samples. Unless HIV genotyping is available to closely monitor epidemiological HIV resistance to TDF, its efficacy as second-line therapy will be greatly compromised.

Provision of antiretroviral therapy (ART) in resource-poor settings employing a public health approach has achieved major successes, saving thousands of lives and averting new HIV infections. Recently, ART initiation in all adults living with HIV disregarding $CD4^+$ cell count was recommended for the first time in World Health Organization (WHO) HIV treatment guidelines [1]. However, the ART arsenal available to most resource-poor settings remains limited, and treatment follow-up rarely includes virological monitoring. In this context, antiretroviral resistance remains a major threat to the public health efforts to eradicate the HIV pandemic.

Tenofovir disoproxyl fumarate (TDF), in combination with lamivudine (3TC)/emtricitabine (FTC) and nevirapine (NVP)/efavirenz (EFV), remains an antiretroviral of choice for first-line ART in Africa, being included in the South African national HIV/AIDS treatment plan for naïve patients [2]. Tenofovir has high antiviral potency, allows once-daily dosing (frequently co-formulated) and is well tolerated. However, its efficacy is diminished in the presence of the K65R mutation [3]. Subtype C, the most prevalent subtype in South Africa, selects for this mutation faster than other subtypes due to subtype-specific pathways [4,5]. This is an important concern because failure to TDF-containing regimens is often associated with additional resistance to nucleoside and nonnucleoside reverse transcriptase inhibitors (NRTI and NNRTI). Therefore, patients developing virological failure to TDF might potentially loose multiple second-line ART options.

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Using Sanger sequencing, previous studies reported the emergence of K65R mutation in 23–69.7% of participants developing virological failure to first-line TDF regimens [6–9]. The true prevalence of TDF resistance, however, might have been underestimated by the lack of sensitivity of standard Sanger-based genotyping methods. Achieving a precise estimate of TDF resistance after virological failure of first-line TDF regimens is also key to inform public policy as to whether TDF might be reused in second-line ART or subsequent regimens. Transmission of TDF resistance might also potentially compromise the efficacy of PrEP strategies [1].

To evaluate the prevalence of TDF resistance using ultrasensitive sequencing methods, we developed a retrospective reanalysis of participants developing virological failure to TDF within a larger cohort study conducted at the McCord Hospital, Durban, South Africa. All participants developing virological failure to first-line ART including TDF + 3TC plus an NNRTI received a genotypic resistance test using a validated in-house Sanger-based sequencing assay in Durban, South Africa. Plasma samples from those with no K65R mutation by Sanger sequencing were reanalyzed at the irsiCaixa AIDS Research Institute in Badalona, Spain using MiSeq Illumina (Illumina Inc., California, USA).

In brief, the complete *pol* gene was amplified and sequenced in a MiSeq platform using a Nextera-XT shotgun approach. A 1% threshold level was chosen for detection of minority variants. Resistance mutations were defined according to the IAS-USA 2013 list. Drug susceptibility results were defined according to Stanford HIV Drug Resistance database, and were classified following the susceptible-intermediate-resistant (SIR) code.

Out of 158 participants included in the McCord cohort at the time of this analysis, 88 participants (55.7%) had developed virological failure to TDF-including regimens. PCR amplification failed in nine samples (10.2%) leaving 79 evaluable patients.

Sanger sequencing detected K65R mutation in 47 out of 79 samples (59.5%). Deep sequencing was attempted in the remaining 32 samples. However, five out of 32 samples had been depleted of volume and could not be evaluated further. K65R mutation was found in eight of the 27 samples evaluable by MiSeq (29.6%) at frequencies in the virus population in the range 1.3–32.5%. Considering the Sanger and deep sequencing results together and assuming that none of the five patients not evaluable by

Table 1. Antiretroviral drug resistance at virological failure of tenofovir disoproxyl fumarate (TDF)-containing first-line first-line antiretroviral therapy by Sanger and ultrasensitive HIV genotyping.

Patient ID	IAS-2013 mutations detected by Sanger sequencing	Additional mutations by Illumina (frequency in the virus population, %)	Changes in drug susceptibility with MiSeq compared with Sanger
1	D67N; M184I; V90I; V179E; Y181C; H221Y	G190A (4.74); K70E (4.5); P225H (8); V106I (9.4)	TDF (S→I); ETR (I→R); RPV (I→R)
2	D67N; K70E; M184V; A98G; K103N; V106M	K65R (20.3); L100I (2.1); Y181C (16,1)	TDF (I→R); ETR (I→R); RPV (I→R)
3	M184V; V106M; G190A	D67N (1.3); K103N (16.7); K65R (27.8); M184I (29.4); M230L (28.1)	TDF (S→R); RPV (I→R)
4	M184V; T215Y; V106M; Y188L	D67N (1.9); G190A (13.6); K101E (12.5)	TDF (S→I); ETR (S→I)
5	M184V; K103N; V108I	A62V (2.8); D67N (4.2); P225H (2.4)	AZT (S→I)
6	M184V; K103N; V106M	D67N (1.3); M230L (31.8)	ETR (S→I); RPV (S→I)
7	M184I; V90I; Y181C; H221Y	A98G (3.2); G190A (9.5); M184I (14.6); V179D (11.6)	ETR (I→R); RPV (I→R)
8	K70E; M184V; V90I; K103N; E138G	K65R (1.3)	TDF (I→R)
9	M184V; V106M; V108I; E138A; G190A	H221Y (1.6); K219E (2.7); K70E (8.6); L74V (3.2); V90I (8.8); Y115F (35.7)	TDF (S→I)
10	M184V; K103N; V106M	K103S (2.4)	
11	M184I; V90I; Y181C; K101E	M184V (9.9)	
12	V106M	No additional mutations found	
13	M184V; V106M; V179D	No additional mutations found	
14	K103N; P225H	V90I (1.2)	
15	No mutations found	Y188C (27.7)	
16	No mutations found	No additional mutations found	
17	Y115F; V106M; Y188C	A62V (2.4)	
18	D67G; T69N; K101E; V106M; H221Y	K65R (4.9)	TDF (S→R); AZT (S→I); 3TC (S→I); FTC (S→I)
19	M184V; A98AG; K103RST; G190A	K65R (32.5); V108I (9.8); Y181C (12.5)	TDF (S→R); ETR (S→R); RPV (I→R);
20	M184I	No additional mutations found	
21	K103N; M184V; P225H	K65R (30.5); K70E (16.4); A98G (5.8); L100I (10.8); V108I (1.5); K219Q (5.1)	TDF (S→R); ETR (S→I); RPV (I→R)
22	M184I; V106M; V179D; M230L	K65R (17.5); K70E (16.7); L74V (15.9); H221Y (4.1); F227C (29.4)	
23	M184V; V106M; G190A; F227L	Y115F (1.4)	
24	M184V; K103N; V108IV; P225HP	No additional mutations found	
25	M184V; K103N; G190A	D67N (4.6); K103S (33.1); E138G (1.2)	
26	D67N; K70E; M184V; V90IV; K101E; V106M; G190A; F227L	K103N (3.8); V179D (9.2); H221Y (1.4)	RPV (I→R)
27	M184V; V106M; V179D	A62V (12.1); K65R (11.8)	TDF (S→R)

3TC, lamivudine; AZT, zidovudine; ETR, etravirine; FTC, emtricitabine; RPV, rilpivirine.

MiSeq had the K65R mutation, a conservative estimate of the overall prevalence of K65R mutation was 69.6%, which is a 10.1% increase in prevalence relative to Sanger sequencing. Prevalence was calculated using only TDF-failing and PCR-success patients.

In addition, deep sequencing detected IAS-USA mutations missed by Sanger in 22 out of 27 patients (81.4%) at frequencies in the range 1.1 – 35.7% in the virus population (Table 1). Such additional mutations changed the predicted drug susceptibility in 15 out of 27 patients (55.5%), mostly affecting TDF, etravirine (ETR) and rilpivirine (RPV), although the predicted susceptibility to NVP or EFV was not affected (Table 1). According to deep sequencing data, 21/27 (77.7%), 25/27 (92.6%), 13/27 (48.1%) and 15/27 (55.5%) were resistant to 3TC/FTC, NVP/EFV, ETR and RPV, respectively, whereas only three participants (11.1%) had intermediate resistance to zidovudine (AZT) – only one (3%) by Sanger sequencing.

Our findings confirm initial estimations that TDF might lose antiviral efficacy in virtually all patients infected with a subtype C HIV developing virological failure to this drug. Thereby, unless HIV genotyping is available to ensure that HIV remains susceptible to TDF, the use of this drug will be greatly compromised in efficacy for second-line therapy, and should not be prescribed except if no other treatment options are available. Continued surveillance of primary resistance in Africa is key to survey transmission of TDF-resistant mutants to newly HIV-infected patients, which could impact the efficacy of both first-line ART and PrEP [10,11]. To date, rates of virological failure to first-line TDF regimens and transmission of K65R mutants have remained low according to Sanger sequencing estimates [12,13]. The fitness cost of the K65R mutation, however, makes K65R mutants wane and thus might be missed by Sanger methods.

Another remarkable finding of our study was that, in addition to identifying K65R, additional resistance mutations detected with MiSeq relative to Sanger mainly affected the predicted susceptibility to the second-generation NNRTIs ETR and RPV, but did not largely influence viral susceptibility to other antiretrovirals, including AZT. On the one hand, this suggests that ETR and RPV might not be good options for second-line ART regimens following EFV or NVP failure. On the other hand, our findings support AZT as a second-line drug in South Africa, used in combination with 3TC and LPV or other protease inhibitors [2] or even integrase inhibitors. Whereas routine drug resistance testing may help decide which NRTIs to use in second-line therapy, the EARNEST trial recently showed that, even without this information, second-line regimens including boosted protease inhibitor plus two NRTIs retained better virological outcomes than protease inhibitor monotherapy, even in the presence of high-level resistance to the NRTI backbone [14], suggesting that residual NRTI activity may be sufficient when combined with highly potent boosted protease inhibitor-based therapy.

Despite its limitations – including a small sample size, lack of adherence data and the inclusion of patients under clinical care which might not represent the general South Africa population – this study not only confirms the development of TDF resistance in most patients developing TDF failure in South Africa, but also supports current public health algorithms for HIV clinical management.

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Conflicts of interest

There are no conflicts of interest.

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References

1. World Health Organization. Guideline on when to start antiretroviral therapy and on preexposure prophylaxis for HIV. Geneva: WHO; 2015 http://www.sahivsoc.org/upload/documents/ART%20Guidelines%2015052015.pdf.
2. National Consolidated Guidelines for the prevention of mother-to-child transmission of HIV (PMTCT) and the management of HIV in children, adolescents and adults. 2015.
3. Margot NA, Isaacson E, McGowan I, Cheng AK, Schooley RT, Miller MD. **Genotypic and phenotypic analyses of HIV-1 in antiretroviral-experienced patients treated with tenofovir DF.** *AIDS* 2002; **16**:1227–1235.
4. Brenner BG, Oliveira M, Doualla-Bell F, Moisi DD, Ntemgwa M, Frankel F, *et al*. **HIV-1 subtype C viruses rapidly develop K65R resistance to tenofovir in cell culture.** *AIDS* 2006; **20**:F9–13.
5. Invernizzi CF, Coutsinos D, Oliveira M, Moisi D, Brenner BG, Wainberg MA. **Signature nucleotide polymorphisms at positions 64 and 65 in reverse transcriptase favor the selection of the K65R resistance mutation in HIV-1 subtype C.** *J Infect Dis* 2009; **200**:1202–1206.
6. Van Zyl GU, Liu TF, Claassen M, Engelbrecht S, de Oliveira T, Preiser W, *et al*. **Trends in genotypic HIV-1 antiretroviral resistance between 2006 and 2012 in South African patients receiving first- and second-line antiretroviral treatment regimens.** *PLoS One* 2013; **8**:e67188.
7. Hoffmann CJ, Ledwaba J, Li J-F, Johnston V, Hunt G, Fielding KL, *et al*. **Resistance to tenofovir-based regimens during treatment failure of subtype C HIV-1 in South Africa.** *Antivir Ther* 2013; **18**:915–920.
8. Sunpath H, Wu B, Gordon M, Hampton J, Johnson B, Moosa M-YS, *et al*. **High rate of K65R for antiretroviral therapy-naive patients with subtype C HIV infection failing a tenofovir-containing first-line regimen.** *AIDS* 2012; **26**:1679–1684.
9. Skhosana L, Steegen K, Bronze M, Lukhwareni A, Letsoalo E, Papathanasopoulos MA, *et al*. **High prevalence of the K65R mutation in HIV-1 subtype C infected patients failing tenofovir-based first-line regimens in South Africa.** *PLoS One* 2015; **10**:e0118145.
10. Margot NA, Lu B, Cheng A, Miller MD. **Resistance development over 144 weeks in treatment-naive patients receiving tenofovir disoproxil fumarate or stavudine with lamivudine and efavirenz in Study 903.** *HIV Med* 2006; **7**:442–450.
11. McColl DJ, Margot NA, Wulfsohn M, Coakley DF, Cheng AK, Miller MD. **Patterns of resistance emerging in HIV-1 from antiretroviral-experienced patients undergoing intensification therapy with tenofovir disoproxil fumarate.** *J Acquir Immune Defic Syndr* 2004; **37**:1340–1350.
12. Liu TF, Shafer RW. **Web resources for HIV type 1 genotypic-resistance test interpretation.** *Clin Infect Dis* 2006; **42**:1608–1618.
13. Rhee S-Y, Blanco JL, Jordan MR, Taylor J, Lemey P, Varghese V, *et al*. **Geographic and temporal trends in the molecular epidemiology and genetic mechanisms of transmitted HIV-1 drug resistance: an individual-patient- and sequence-level meta-analysis.** *PLoS Med* 2015; **12**:e1001810.
14. Paton NI, Kityo C, Hoppe A, Reid A, Kambugu A, Lugemwa A, *et al*. **Assessment of second-line antiretroviral regimens for HIV therapy in Africa.** *N Engl J Med* 2014; **371**:234–247.

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Short Communication

No detection of the NS5B S282T mutation in treatment-naïve genotype 1 HCV/HIV-1 coinfected patients using deep sequencing

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ABSTRACT

Background: The S282T mutation is the main variant described associated with resistance to nucleos(t)ide analogues hepatitis C virus (HCV) NS5B polymerase inhibitors.
Objective: We aimed here to investigate whether this substitution pre-existed in treatment naive HCV/HIV-1 coinfected patients.
Study design: NS5B polymerase deep sequencing was performed at a median coverage per base of 4471 in 16 patient samples.
Results: No S282T variant was detected in the 16 analyzed samples.
Conclusion: This finding is in agreement with the high genetic barrier of nucleoside analogues NS5B polymerase inhibitors and the clinical efficacy of these compounds.

1. Background

The approval of two hepatitis C virus (HCV) protease inhibitors, telaprevir and boceprevir, in combination with pegylated interferon plus ribavirin (pegIFN/RBV) has improved sustained virological response rates in patients infected with HCV genotype 1 [1–3]. However, viral breakthroughs due to the selection of HCV variants resistant to the administered protease inhibitors have been reported [4]. Not surprisingly, minority HCV NS3 protease inhibitor resistance mutations were also reported in treatment-naive HCV genotype 1 monoinfected [5–8] and human immunodefiency virus type 1 (HIV-1) coinfected patients [9,10].

The estimated median in vivo HCV mutation rate is 2.5×10^{-5} mutations per nucleotide per genome replication [11]; this, combined with a virion production and clearance rate of 10^{12} virions per day [12], suggests that resistant HCV variants are likely to preexist and rapidly outgrow wild type virus under drug selective pressure. The two NS5B nucleos(t)ide analogues inhibitors that more quickly are advancing to the clinic, mericitabine and sofosbuvir, have been reported to select in vitro substitutions at position S282 which is in close vicinity to the enzyme's catalytic site. The S282T substitution has been reported to confer a 3–6-fold loss of in vitro sensitivity to mericitabine [13]. This substitution results in a moderate loss of antiviral activity but in a large reduction in replicative capacity [14]. However, no baseline population sequencing analysis with predicted resistance to NS5B nucleos(t)ides analogues have been detected in vivo in genotype 1 infected patients [8,15]. The S282T mutation has been found to be dominant only in a genotype 4a isolate [16]. Similarly, classical clonal analysis of the viral quasispecies indicated that mutation S282T was not present at baseline in treatment-naïve genotype 1 HCV infected patients [17]. Moreover, the S282T mutation has been extremely difficult to detect in vivo even in patients with failure to mericitabine or sofosbuvir [4]. It is notable that most failures were relapses after therapy; no viral breakthroughs.

2. Objective

To further explore the prevalence of S282T, we investigated here whether the NS5B S282T minor variant preexisted in HCV/HIV-1 coinfected patients. HIV-1 coinfected patients have both higher HCV viral and higher rates of treatment failure than HCV monoinfected patients. To this end, NS5B deep sequencing was performed in samples from treatment naïve genotype 1 HCV/HIV-1 coinfected patients.

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Table 1
Clinical characteristics of study patients.

Patient	Gender	HCV RNA[a]	HCV genotype	AST[b]	ALT[b]	CD4+ cell count[c]	HIV RNA[d]
1	M	5.68	1b	40	78	630	<80
2	M	6.39	1b	76	200	272	<80
3	M	5.939	1b	28	15	307	<80
4	F	5.83	1b	58	52	350	<80
5	M	5.85	1b	89	208	785	6600
6	F	5.92	1b	53	89	342	<80
7	M	5.70	1b	51	56	247	3300
8	F	6.28	1b	33	31	400	<80
9	M	5.93	1b	65	111	308	<80
10	M	6.756	1a	110	116	5236	<80
11	M	5.85	1a	49	114	611	<80
12	F	5.505	1a	28	26	507	<80
13	M	5.92	1a	42	59	475	4900
14	F	6.50	1a	38	34	660	<80
15	M	5.67	1a	58	103	747	<80
16	M	5.74	1a	59	95	661	<80

[a] (IU/mL) ($\log_{10}$).
[b] (U/L).
[c] CD4+ cell counts/μL.
[d] Copies/mL.

3. Study design

3.1. Patients

Sixteen HCV/HIV-1 coinfected patients were studied (Table 1). All patient samples were infected with HCV of genotype 1a or 1b and were naive for pegIFNa/RBV treatment and direct acting antivirals (DAAs). The median HCV viral load was 5.88 (log 10) IU/mL and the interquartile range was 5.72–6.10 (log 10) IU/mL. All HIV-1-co-infected patients were receiving antiretroviral therapy.

3.2. Methods

HCV RNA was extracted from 1 mL of the plasma samples by ultracentrifugation and purified by using the QIAamp viral RNA minikit (QIAGEN) according to the manufacturer's instructions. Three independent RT-PCRs (RT-PCR One Step Superscript III/Platinum HF Taq) (Invitrogen) were carried out for each sample with oligonucleotides NS5B-F1 (5′-TCTCAGCGACGGGTCWTGGTC-3′, H77 positions 7526–7546) and NS5B-R1 (5′-CCTGCAGMAAGYAGGAGTAGGC-3′, H77 positions 9303–9324) using standard conditions. Amplicon libraries were generated from first-round PCR products. These amplicons incorporated adaptators A and B, and identifiers used in parallel sample sequencing needed for bidirectional 454 sequencing. Nested PCR was performed with Platinum Taq High Fidelity (Life Technologies) and oligonucleotides NS5B-F3 (5′-TCTACCAATGTTGTGACYTGG-3′, H77 positions 8305–8326) and NS5B-R3 (5′-GCATCGTGCAGTCCTGGAGC-3′, H77 positions 8510–8529). The HCV 1b replicon plasmid (I389/NS3-3′/LucUbiNeo-ET) was retrotranscribed to RNA (Ambion, Life Technologies) and tested in parallel with patient samples to determine the assay background. Nested PCR products were purified using AMPure Magnetic Beads (Beckman Coulter). Concentration and quality of purified PCR products were determined using fluorometry (PicoGreen, Life Technologies) and spectrophotometry (Lab on a Chip, Agilent Technologies). Equimolar amplicon pools were made to perform emPCR, adding a ratio of 1:1 between molecules and 454-beads. The sequence platform used was Genome System Junior (Life Sequencing/Roche). Amplicon Variant Analyzer (AVA software v2.6) was employed to analyze and obtain sequence alignments. Error corrected consensus sequences, as obtained from AVA, were used for amino acid variant calling. Variants were considered valid when present in both forward and reverse directions.

```
          246   *       260         *       280         *       300
1b.I389 : APEARQAIRSLTERLYIGGPLTNSKGQNCGYRRCRASGVLTTSCGNTLTCYLKAAAACRAAK :  62
1       : ....K.....................................................S..... :  62
2       : ....K.....................................................T..... :  62
3       : ........K.................................................S..... :  62
4       : ..........................................................T..... :  62
5       : ........K.................................................T..... :  62
6       : ..........................................................S..... :  62
7       : ..........................................................S..... :  62
8       : ..........................................................S..... :  62
9       : ..........................................................S..... :  62
1a.H77  : D.Q..V..K.......V.......R.E.......................I..R......G :  62
10      : D.Q..V..K.......V.......R.E.......................I..Q......G :  62
11      : D.Q..V..K.......V.......R.E.......................I..Q......G :  62
12      : D.Q..V..K.......V.......R.E.......................I..Q......G :  62
13      : D.Q..V..K.......V.......R.E.......................I..Q......G :  62
14      : D.Q..V..K.......V.......R.E.......................I..Q......G :  62
15      : D.Q..V..K.......V.......R.E.......................I..Q......G :  62
16      : D.Q..V..K.......V.......R.E.......................I..Q......G :  62
```

Fig. 1. Alignment of the HCV NS5B amino acid consensus sequences of the 16 study patients compared with prototype 1b (I389/NS3-3′/LucUbiNeo-ET) and prototype 1a (H77) sequences. Dots indicate amino acid sequence identity.

Table 2
HCV NS5B amino acid substitutions compared with prototype 1b (I389/NS3-3′/LucUbiNeo-ET) and 1a (H77) sequences.

Patient[a]	Amino acid position	Prototype amino acid	Amino acid substitution	Frequency (%)[b]
1	250	R	K	99.3
	300	A	S	79.4
	300	A	T	19.8
2	250	R	K	99.4
	293	L	P	0.7
	300	A	T	100
3	254	R	K	99.9
	300	A	S	100
4	254	R	K	32.8
	260	L	F	0.6
	267	T	I	0.6
	300	A	T	99.4
5	254	R	K	100
	300	A	T	100
6	249	A	S	0.9
	281	A	T	1.0
	300	A	S	100
7	250	R	K	1.1
	300	A	S	98.6
	300	A	T	1.4
8	300	A	S	100
10	252	A	V	1.2
	300	R	Q	100
11	251	V	M	6.0
	300	R	Q	99.9
	305	A	T	0.6
12	300	R	Q	99.9
	282	S	G	0.8
	305	A	T	1.0
13	300	R	Q	100
14	252	A	V	5.0
	262	V	I	0.6
	300	R	Q	99.8
15	300	R	Q	75.4
16	300	R	Q	99.8

[a] Genotype 1b sequences were compared to prototype 1b (I389/NS3-3′/LucUbiNeo-ET) sequence and genotype 1a sequences were compared to prototype 1a (H77) sequence.
[b] Only frequencies above 0.5% are shown.

4. Results and discussion

Deep sequencing results of the NS5B coding region were obtained for the 16 patient samples and genotype 1b replicon control. A total of 76,007 sequences were obtained after quality checks. The resultant sequences were used to generate a list of mutations present in the tested samples. On average, 4471 reads were obtained per each nucleotide position of the 184 nucleotides sequenced. To establish the background noise derived from PCR amplification and 454 pyrosequencing, the genotype 1b replicon control was sequenced, with an average coverage of 1385 reads per nucleotide position. The background noise for replicon control was below 0.5% nucleotide substitutions per NS5B nucleotide position. The median Shannon entropy was 0.01402 ± 0.00475 (SD) and interquartile range was 0.02568–0.00496. Shannon Entropy for genotype 1b replicon control was 0.00093 ± 0.00000. Patient amino acid sequences were compared with the corresponding prototype 1b (I389/NS3-3′/LucUbiNeo-ET) or 1a (H77) sequences (Fig. 1). When patient amino acid sequences were compared with the corresponding prototype sequences, 17 amino acids (27%) were altered and 13 (21%) showed mixed variants (Table 2). Interestingly, a variant S282G was detected in patient 12. However, the NS5B S282T mutation was not detected in any of the samples analyzed in our study. S282G was likely generated through a transition mutation, while S282T is generated though a transversion. Transitions are much more frequent than transversions, which provides a possible mechanism for its appearance and detection.

These findings extend and confirm previous studies which showed the low prevalence in vivo of the S282T mutation [18]. In addition, our results may explain the absence of breakthroughs in patients failing therapy with NS5B nucleos(t)ide analogues and their high vivo barrier for the development of resistance. Remarkably, viral breakthrough has been rarely observed even in patients on nucleos(t)ide analogue monotherapy. Nevertheless, other mechanisms cannot be discarded to explain the absence of breakthroughs in patients that fail therapy with NS5B nucleos(t)ide analogues because non-mutant HCV in some patients may resist the treatment. Therefore, absence of the S282T mutation may not be the only indicator of sensitivity to the therapy. In contrast, abundant NS3 protease inhibitor resistant mutants have been detected by deep sequencing in patients undergoing NS3 protease inhibitor monotherapy or in baseline treatment-naïve patient samples [19,20]. Most of samples of this study had minor NS3 protease inhibitor resistant mutants when they were analyzed by classical clonal analysis [9]. Furthermore, it is also possible to detect minority NS3 protease inhibitor resistant mutants even

in acute hepatitis C patients which have less nucleotide quasispecies diversity when compared to chronic infected patients (data not shown). Pretreatment minority resistance substitutions to daclatasvir, an NS5A replication complex inhibitor, have been also described [21]. This study therefore indicates the differences between the prevalence of NS5B nucleoside analogue inhibitor resistance and other HCV DAAs. A possible explanation for the low prevalence of the S282T mutation is the lethality of this substitution.

Although this study provides compelling evidence for the low prevalence of NS5B S282T mutants in naïve samples, our results may be limited by absence of samples from patients failing therapy with mericitabine or sofosbuvir. Further work should therefore include deep sequencing analysis of samples from relapsers failing therapy with nucleos(t)ide analogues inhibitors in order to evaluate whether the S282T mutation is lethal in most genotype 1 NS5B backgrounds.

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Conflict of interest

The authors declare no conflict of interest.

Ethical approval

Ethical approval was given by committee of Hospital Universitari Germans Trias i Pujol under the reference number E0-10-008.

References

[1] Jacobson IM, McHutchison JG, Dusheiko G, Di Bisceglie AM, Reddy KR, Bzowej NH, et al. Telaprevir for previously untreated chronic hepatitis C virus infection. N Engl J Med 2011;364:2405–16.
[2] Bacon BR, Gordon SC, Lawitz E, Marcellin P, Vierling JM, Zeuzem S, et al. Boceprevir for previously treated chronic HCV genotype 1 infection. N Engl J Med 2011;364:1207–17.
[3] Poordad F, McCone Jr J, Bacon BR, Bruno S, Manns MP, Sulkowski MS, et al. Boceprevir for untreated chronic HCV genotype 1 infection. N Engl J Med 2011;364:1195–206.
[4] Wyles DL. Antiviral resistance and the future landscape of hepatitis C virus infection therapy. J Infect Dis 2013;207(Suppl. 1):S33–9.
[5] Bartels DJ, Zhou Y, Zhang EZ, Marcial M, Byrn RA, Pfeiffer T, et al. Natural prevalence of hepatitis C virus variants with decreased sensitivity to NS3.4A protease inhibitors in treatment-naive subjects. J Infect Dis 2008;198:800–7.
[6] Chary A, Winters MA, Kottilil S, Murphy AA, Polis MA, Holodniy M. Impact of interferon-ribavirin treatment on hepatitis C virus (HCV) protease quasispecies diversity in HIV- and HCV-coinfected patients. J Infect Dis 2010;202:889–93.
[7] Cubero M, Esteban JI, Otero T, Sauleda S, Bes M, Esteban R, et al. Naturally occurring NS3-protease-inhibitor resistant mutant A156T in the liver of an untreated chronic hepatitis C patient. Virology 2008;370:237–45.
[8] Kuntzen T, Timm J, Berical A, Lennon N, Berlin AM, Young SK, et al. Naturally occurring dominant resistance mutations to hepatitis C virus protease and polymerase inhibitors in treatment-naive patients. Hepatology 2008;48:1769–78.
[9] Aparicio E, Franco S, Parera M, Andres C, Tural C, Clotet B, et al. Complexity and catalytic efficiency of hepatitis C virus (HCV) NS3 and NS4A protease quasispecies influence responsiveness to treatment with pegylated interferon plus ribavirin in HCV/HIV-coinfected patients. J Virol 2011;85:5961–9.
[10] Franco S, Bellido R, Aparicio E, Canete N, Garcia-Retortillo M, Sola R, et al. Natural prevalence of HCV minority variants that are highly resistant to NS3/4A protease inhibitors. J Viral Hepat 2011;18:e578–82.
[11] Ribeiro RM, Li H, Wang S, Stoddard MB, Learn GH, Korber BT, et al. Quantifying the diversification of hepatitis C virus (HCV) during primary infection: estimates of the in vivo mutation rate. PLoS Pathog 2012;8:e1002881.
[12] Neumann AU, Lam NP, Dahari H, Gretch DR, Wiley TE, Layden TJ, et al. Hepatitis C viral dynamics in vivo and the antiviral efficacy of interferon-alpha therapy. Science 1998;282:103–7.
[13] Ali S, Leveque V, Le Pogam S, Ma H, Philipp F, Inocencio N, et al. Selected replicon variants with low-level in vitro resistance to the hepatitis C virus NS5B polymerase inhibitor PSI-6130 lack cross-resistance with R1479. Antimicrob Agents Chemother 2008;52:4356–69.
[14] Ludmerer SW, Graham DJ, Boots E, Murray EM, Simcoe A, Markel EJ, et al. Replication fitness and NS5B drug sensitivity of diverse hepatitis C virus isolates characterized by using a transient replication assay. Antimicrob Agents Chemother 2005;49:2059–69.
[15] Bartels DJ, Sullivan JC, Zhang EZ, Tigges AM, Dorrian JL, De Meyer S, et al. Hepatitis C virus variants with decreased sensitivity to direct-acting antivirals (DAAs) were rarely observed in DAA-naive patients prior to treatment. J Virol 2013;87:1544–53.
[16] Newman RM, Kuntzen T, Weiner B, Berical A, Charlebois P, Kuiken C, et al. Whole genome pyrosequencing of rare hepatitis C virus genotypes enhances subtype classification and identification of naturally occurring drug resistance variants. J Infect Dis 2013;208:17–31.
[17] Le Pogam S, Yan JM, Chhabra M, Ilnicka M, Kang H, Kosaka A, et al. Characterization of hepatitis C virus (HCV) quasispecies dynamics upon short-term dual therapy with the HCV NS5B nucleoside polymerase inhibitor mericitabine and the NS3/4 protease inhibitor danoprevir. Antimicrob Agents Chemother 2012;56:5494–502.
[18] Margeridon S, Le Pogam S, Liu TF, Hanczaruk B, Simen BB, Shulman N, et al. No detection of variants bearing NS5B S282T mericitabine (MCB) resistance mutation in DAA treatment-naive HCV genotype 1 infected patients using ultra-deep pyrosequencing (UDPS). Hepatology 2011;54:532A.
[19] Svarovskaia ES, Martin R, McHutchison JG, Miller MD, Mo H. Abundant drug-resistant NS3 mutants detected by deep sequencing in hepatitis C virus-infected patients undergoing NS3 protease inhibitor monotherapy. J Clin Microbiol 2012;50:3267–74.
[20] Fonseca-Coronado S, Escobar-Gutierrez A, Ruiz-Tovar K, Cruz-Rivera MY, Rivera-Osorio P, Vazquez-Pichardo M, et al. Specific detection of naturally occurring hepatitis C virus mutants with resistance to telaprevir and boceprevir (protease inhibitors) among treatment-naive infected individuals. J Clin Microbiol 2012;50:281–7.
[21] McPhee F, Hernandez D, Yu F, Ueland J, Monikowski A, Carifa A, et al. Resistance analysis of hepatitis C virus genotype 1 prior treatment null responders receiving daclatasvir and asunaprevir. Hepatology 2013;58:902–11.

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