

# HIV-1 integrase genotyping is reliable and reproducible for routine clinical detection of integrase resistance mutations even in patients with low-level viraemia

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**Objectives:** Integrase drug resistance monitoring deserves attention because of the increasing number of patients being treated with integrase strand-transfer inhibitors. Therefore, we evaluated the integrase genotyping success rate at low-level viraemia (LLV, 51–1000 copies/mL) and resistance in raltegravir-failing patients.

**Methods:** An integrase genotypic resistance test (GRT) was performed on 1734 HIV-1 samples collected during 2006–13. Genotyping success rate was determined according to the following viraemia levels: 51–500, 501–1000, 1001–10000, 10001–100000 and >100000 copies/mL. The reproducibility of integrase GRT was evaluated in 41 plasma samples processed in duplicate in two reference centres. The relationship between LLV and resistance prevalence was evaluated in a subset of 120 raltegravir-failing patients.

**Results:** Overall, the integrase genotyping success rate was 95.7%. For viraemia levels 51–500 and 501–1000 copies/mL, the rate of success was 82.1% and 94.0%, respectively. GRT was reproducible, producing sequences with a high similarity and an equal resistance profile regardless of the sequencing centre or viraemia level. Resistance was detected both at LLV and at viraemia >1000 copies/mL (51–500 copies/mL = 18.2%; 501–1000 = 37.5%; 1001–10000 = 53.7%; 10001–100000 = 30.0%; and >100000 = 30.8%). At viraemia ≤500 copies/mL, Q148H/K/R and N155H had the same prevalence (9.1%), while the Y143C/H/R was completely absent. At early genotyping (within 3 months of raltegravir treatment), Q148H/K/R and N155H mutations were detected regardless of the viraemia level, while Y143C/H/R was observed only in samples with viraemia >1000 copies/mL.

**Conclusions:** Our findings prove the reliability of HIV-1 integrase genotyping and reinforce the concept that this assay may be useful in the management of failures even at LLV.

**Keywords:** integrase, HIV-1 genotyping, low-level viraemia, raltegravir, elvitegravir, dolutegravir, INSTI, drug resistance

## Introduction

Over the past 6 years, raltegravir, the first approved integrase strand-transfer inhibitor (INSTI), has taken on an important role in treating HIV infection, both in antiretroviral-naïve and -experienced patients.<sup>1–4</sup> Nowadays, with two other available INSTIs (elvitegravir and the second-generation inhibitor dolutegravir), this drug class has strengthened combined ART (cART).<sup>5–10</sup> These drugs are suitable for the majority of HIV-1-infected patients because of their potency and tolerability.

Indeed, beyond raltegravir and dolutegravir,<sup>2,9,10</sup> elvitegravir was recently approved by the US FDA for the treatment of HIV infection in adults who are already taking or have taken HIV medicines.<sup>11,12</sup> Moreover, recent evidence demonstrated that INSTIs might be a suitable alternative in virologically suppressed patients in whom a regimen modification or simplification is being considered.<sup>3,13,14</sup> Finally, among seven regimens recommended for treating antiretroviral-naïve patients, four contain INSTIs.<sup>15</sup>

However, raltegravir and elvitegravir have shown a low/intermediate genetic barrier for drug resistance development.<sup>3,16–19</sup>

By contrast, dolutegravir has shown a high genetic barrier to the development of resistance *in vitro* and *in vivo*, in clinical trials,<sup>5,10</sup> but few data about the usage of this drug are available in clinical practice.

Standard-of-care management recommends the use of genotypic testing for INSTI resistance for patients with a previous exposure to this drug class who are starting a new INSTI-based regimen.<sup>15,20–22</sup> In drug-naïve individuals, an INSTI integrase genotypic resistance test (GRT) should be performed if INSTI resistance transmission is a concern.<sup>15,20–22</sup>

In general, GRT is recommended in patients with viraemia levels >500–1000 copies/mL.<sup>15,22</sup>

This restriction of GRT mostly derives from the technical difficulty of many laboratories in obtaining consistent results with samples at low-level viraemia (LLV). However, several findings now support the use of protease/reverse transcriptase GRT at LLV,<sup>23–26</sup> but few data about success and reliability in performing integrase GRT are currently available.<sup>27</sup>

Moreover, it is now widely demonstrated that the emergence of mutations associated with resistance to PIs and reverse transcriptase inhibitors is also found at LLV.<sup>25,26,28</sup>

This phenomenon has great clinical importance since it has been associated with an increased risk of virological failure.<sup>26,28,29</sup> Mutations at LLV can also occur in patients receiving an INSTI-containing regimen.<sup>29,30</sup>

For this reason, integrase resistance monitoring at LLV deserves particular attention because of the potential future extensive use of INSTIs. Indeed, particular attention should be addressed to raltegravir and elvitegravir in first-line treatment because of their low/intermediate genetic barrier to the development of resistance.

In this study, we provide data supporting the reliability and usefulness of integrase GRT at viraemia levels <501–1000 copies/mL by analysing a large population of HIV-1 patients followed in central Italy, who underwent integrase GRT in routine clinical practice. Moreover, we evaluated whether different viraemia levels affect the detection of integrase resistance in HIV-1 patients who failed a raltegravir-containing regimen.

## Patients and methods

### Patients

This retrospective study included 1734 HIV-1 plasma samples with viraemia values >50 copies/mL that were genotyped over the years 2006–13 in two reference centres in Rome (Italy) for routine clinical purposes. Sample information (date of sampling, final results of sequencing, nucleotide sequences obtained and mutations found in each sequence), together with the data of patients for whom genotyping was performed (i.e. viroimmunological, clinical and therapeutic data), were recorded in an anonymous database.

### HIV-1 RNA viral load

Plasma viraemia was determined as previously described<sup>26,31</sup> and samples were stratified into six groups according to the following viraemia ranks: 51–500, 501–1000, 1001–10000, 10001–100000 and >100000 copies/mL.

### HIV-1 integrase sequencing

HIV genotype analysis of the integrase was performed on plasma samples by using either the ViroSeq HIV-1 Integrase Genotyping System (Cela Diagnostics, Alameda, CA, USA) or an in-house assay.

As previously described for HIV-1 protease/reverse transcriptase genotyping,<sup>26</sup> some steps of the ViroSeq HIV-1 integrase module were modified in order to test HIV-1 integrase sequences also in subjects with LLV. Full details on the amplification and sequencing procedures are described in the Supplementary Methods and Figure S1 (available as Supplementary data at JAC Online).

### Subtyping analysis

To define HIV-1 integrase subtypes, sequences were aligned using Clustal W version 2.1<sup>32</sup> and compared with reference sequences for major HIV-1 subtypes and circular recombinant forms (CRFs) (available at the Los Alamos database, <http://www.hiv.lanl.gov>). Subtypes or CRFs were assigned by constructing phylogenetic trees using the Neighbour Joining method. Distances were calculated using MEGA version 5.05 based on the Kimura two-parameter (K2P) model.<sup>33</sup> The reliability of the branching orders was assessed by bootstrap analysis of 1000 replicates. The tree was viewed using the graphical user interface FigTree. Subtype classification was confirmed also by the REGA subtype tool (<http://www.bioafrica.net/rega-genotype/html/subtypinghiv.html>) and the COMET subtype tool (<http://comet.retrovirology.lu/>). To improve the accuracy of recombinant and unique forms, RDP3 software (<http://web.cbio.uct.ac.za/~darren/rdp.html>) and Splits Tree software (<http://en.bio-soft.net/tree/SplitsTree.html>) were used.

### Evaluation of genotyping success rate

The genotyping success rate was determined on the overall population and according to the different viraemia ranks (51–500, 501–1000, 1001–10000, 10001–100000 and >100000 copies/mL), regardless of the genotyping platform upgrades (equipment, kits and reagents) that occurred from 2006 to 2013.

### Evaluation of cross-contamination and resampling of HIV-1 integrase genotyping

To investigate the possibility of cross-contamination or sample mix-up during laboratory procedures, all the HIV-1 integrase sequences obtained from the 1734 genotypic tests requested were used to construct a phylogenetic tree. In particular, among all the sequences (1659 from 1308 patients), 233 were from 85 patients with at least one sequence with contextual viraemia ≤1000 copies/mL and at least one sequence with contextual viraemia >1000 copies/mL. Therefore, the tree was examined to confirm that all sequences from each patient formed separate distinct clusters. Phylogenetic analysis of the HIV-1 integrase sequences was performed by using the K2P model of MEGA version 5.05 with the same parameters as described above.<sup>33</sup>

### Evaluation of the reproducibility of HIV-1 integrase genotyping

The reproducibility of HIV-1 integrase genotyping was evaluated in a subgroup of 41 plasma samples that were processed in duplicate in two reference centres in Rome. Eighteen samples had viraemia ≤1000 copies/mL, while 23 samples had viraemia >1000 copies/mL. The mean genetic distance was used to evaluate the effective reproducibility of HIV-1 integrase genotyping. More specifically, for each pair of sequences obtained from the same patient, the mean genetic distance was calculated by using a K2P model.<sup>33</sup> A maximum likelihood tree was also constructed from these sequences to represent the effective relatedness between the sequences from the same subject. The tree was inferred with the generalized time reversible (GTR) nucleotide substitution model with gamma distribution among site rate heterogeneity, a proportion of invariable sites (GTR+I+Γ<sub>3</sub>) and 1000 bootstrap replicates. Paired samples from the

same subject were identified by a bootstrap support >90%. The analyses were conducted by using MEGA version 5.05 software. Moreover, resistance-associated mutations were evaluated and compared for each couple of sequences.

### Evaluation of INSTI resistance

The evaluation of acquired resistance to INSTIs was carried out on a subset of 182 samples successfully genotyped from 120 patients for whom a GRT was required because of virological failure on a raltegravir-containing regimen (viraemia >50 copies/mL).

Resistance to raltegravir was defined by the presence of at least one primary resistance mutation among Y143C/H/R, Q148H/K/R and N155H. The prevalence of INSTI secondary resistance mutations among V54I, T66A/K/I, L74I/M, E92G/Q/V, T97A, G118R, F121Y, E138A/K, G140A/C/S, S147G, V151A/I/L, N155S/T, E157Q, G163K/R, S230R, D232N and R263K<sup>34-38</sup> was also evaluated. The analyses performed are listed below.

### Evaluation of the prevalence of raltegravir resistance according to different viraemia levels

For this analysis, only one integrase sequence per patient for each viraemia range was considered to avoid overcalling of resistance mutations. In the case where more than one sequence per patient was available, the sequence with the higher number of drug resistance mutations was retained for the analysis. For sequences without resistance, the last one was retained. A total of 156 sequences were analysed.

### Evaluation of cross-resistance to INSTI according to different viraemia levels

To evaluate the cross-resistance among INSTIs in the 156 sequences from patients failing raltegravir, the genotypic susceptibility score (GSS) for raltegravir, elvitegravir and dolutegravir was calculated according to the Stanford algorithm (<http://sierra2.stanford.edu/sierra/servlet/JSierra?action=algSequenceInput>). The GSS was scored as 0 (resistant virus), 0.5 (virus with intermediate resistance) and 1 (susceptible virus). For each drug, the GSS was stratified according to the viraemia level score.

### Evaluation of the emergence of INSTI resistance according to the duration of raltegravir treatment

For this analysis, all the 182 sequences were analysed according to the duration of raltegravir treatment (defined as the time from the first administration of raltegravir to the time of integrase genotyping). The timepoints considered were ≤3, 4–6, 7–9, 10–12 and >12 months. The development of resistance was evaluated by also considering viraemia levels.

### Statistical analysis

Potential differences among the different viraemia groups were evaluated as follows: (i) for categorical variables, by the  $\chi^2$  test for trend and Pearson's  $\chi^2$  test or Fisher's exact test as appropriate; and (ii) for continuous variables, by the Mann-Whitney test or Kruskal-Wallis test as appropriate. In all analyses, *P* values <0.05 were considered as statistically significant. The statistical programs used were R open-source software (version 3.0.2) and SPSS (version 19) for Windows® (SPSS, Chicago, IL, USA).

## Results

### Study population

This study included 1734 plasma samples with viraemia >50 copies/mL from 1358 patients, processed for integrase GRT

**Table 1.** Characteristics of plasma samples with HIV RNA >50 copies/mL at integrase genotypic resistance test

Variables/categories	<i>n</i> (%)
Patients, <i>n</i> = 1358	
with only one sample	1123 (82.7)
with more than one sample	235 (17.3)
Samples, <i>n</i> = 1734	
from drug-naïve patients	392 (22.6)
from drug-experienced INSTI-naïve patients	717 (41.4)
from raltegravir-experienced patients	625 (36.0)
Subtype	
B	1412 (81.4)
CRF02_AG	101 (5.8)
F	69 (4.0)
C	40 (2.3)
BF <sup>a</sup>	35 (2.0)
G	26 (1.5)
A	17 (1.0)
other <sup>b</sup>	34 (2.0)
Samples according to viraemia ranges (copies/mL)	
51–500	168 (9.7)
501–1000	67 (3.9)
1001–10000	370 (21.3)
10001–100000	650 (37.5)
>100000	479 (27.6)

INSTI, integrase strand-transfer inhibitor.

<sup>a</sup>Including: CRF\_12\_BF (17), CRF40\_BF (14), 3 CRF46\_BF (3), CRF47\_BF (1).

<sup>b</sup>Including: CRF01\_AE (15); CRF19\_cpx (3); D (2); CRF06\_cpx (2); CRF09\_cpx (2); JK (1); CRF02\_AG, B (1); CRF02\_AG,F,I,J (1); CRF10\_CD (1); CRF14\_BG (1); CRF18\_cpx (1); CRF22\_01A2 (1); CRF25\_cpx (1); CRF31\_BC (1); CRF35\_AD (1).

in routine clinical practice from 2006 to 2013. Among these 1734 samples, 625 (36.0%), 717 (41.4%) and 392 (22.6%) were obtained from drug-naïve patients, drug-experienced patients never exposed to INSTIs and raltegravir-experienced patients, respectively. Overall, viraemia levels of 51–1000 copies/mL accounted for 235 (13.6%) samples (168 of them with viraemia levels of 51–500 copies/mL) (Table 1).

Phylogenetic analysis revealed that subtype B was the most prevalent strain (81.4%) (Table 1). All the other subtypes were present with prevalence <6%; the most prevalent ones were the recombinant form CRF02\_AG (5.8%) and subtype F (4%) (Table 1).

### Genotyping success rate

The overall success rate of the genotype amplification and sequencing was 95.7% and was comparable in the two clinical centres where the sequences were produced (95.4% versus 96.0%; *P* = 0.542). The rate of success was 94.0% for samples with viraemia levels of 501–1000 copies/mL and it was still relevant at 51–500 copies/mL (82.1%) (Table 2).

The additional use of a nested amplification has significantly improved the overall success rate in samples with LLV. Indeed, the requirement for the nested amplification significantly decreased at higher viraemia levels (51–500 copies/mL: 69.4%;

**Table 2.** HIV-1 genotyping resistance success rate according to different viraemia levels

Viraemia range (copies/mL)	Success rate <sup>a</sup> [n (%)]
Overall (n=1734)	1659 (95.7)
51–500 (n=168)	138 (82.1)
501–1000 (n=67)	63 (94.0)
1001–10000 (n=370)	351 (94.9)
10001–100000 (n=650)	635 (97.7)
>100000 (n=479)	472 (98.5)

<sup>a</sup>The success of the integrase genotypic resistance test in plasma samples from HIV-1-infected patients was evaluated on the overall population with viraemia >50 copies/mL (n=1734) and by stratifying for viraemia range.

500–1000 copies/mL: 55.8%; 1001–10000 copies/mL: 49.6%; 10001–100000 copies/mL: 26.4%; and >100000 copies/mL: 17.3%;  $P<0.001$ ).

A higher success rate was found for sequences from subtype B than those from non-B subtypes (96.7% versus 91.0%;  $P<0.001$ ). This difference in the proportion of successfully performed assays was mainly due to the presence of several recombinant forms (success rate: 96.7% for subtype B versus 86.9% for recombinant forms;  $P<0.001$ ). By focusing attention on the two most prevalent non-B subtypes (F and CRF02\_AG), no difference in the success rate was found between subtypes F and B (F: 97.1%), while a significantly lower success rate was found in CRF02\_AG (88.1%) compared with subtype B ( $P<0.001$ ). By repeating the success rate analysis in the subsets of samples from drug-naïve patients, drug-experienced patients never exposed to INSTIs and raltegravir-experienced patients, a similar rate was observed (data not shown).

### Evaluation of cross-contamination and resampling of HIV-1 integrase genotyping

In order to check for any laboratory contaminations, we performed a phylogenetic analysis on all the sequences obtained from the 1734 genotypic tests requested (1659 sequences from 1308 patients). Some 99% (1643/1659) of the sequences had the expected distribution in the phylogenetic tree. The outgroups of the 16 remaining sequences may be caused by cross-contamination or mistakes in sample/sequence attribution.

Among these 1659 sequences, 233 were from 85 patients with at least one sequence with contextual viraemia  $\leq 1000$  copies/mL and at least one sequence with contextual viraemia >1000 copies/mL. By evaluating each cluster, we found that sequences belonging to the same subject showed a high homology (bootstrap value >90%) in 97.6% of cases (83/85 patients), regardless of LLV. Three examples of clusters containing sequences from three distinct patients with different viraemia values and at different time periods are reported in Figure S2.

### Evaluation of the reproducibility of HIV-1 integrase genotyping

In order to assess the reproducibility of HIV-1 integrase genotyping, 41 plasma samples were processed in duplicate in two

reference centres in Rome. Overall, a high homology among the sequences was observed because of the very low genetic distance found between the HIV-1 integrase sequences from the same patient [median (IQR): 0.00125 (0–0.00376)]. No significant differences were found by comparing the genetic distance among the sequences with viraemia  $\leq 1000$  copies/mL and those with viraemia >1000 copies/mL [0.00125 (0–0.00377) versus 0.00125 (0–0.00251);  $P=0.674$ ], thus confirming a high homology of sequences regardless of the viraemia level. The maximum likelihood tree constructed confirmed the tight relatedness between sequences from the same sample (Figure S3).

Finally, a complete concordance in the detection of resistance-associated mutations between the two sequencing centres was found (data not shown).

### Evaluation of raltegravir resistance according to different viraemia ranges in patients failing raltegravir

The prevalence of raltegravir resistance mutations according to different viraemia levels was analysed in 156 sequences from a subgroup of 120 patients failing raltegravir. The characteristics of this patient subgroup are reported in Table S1. No significant differences were found by stratifying the sequences for viraemia ranges. Overall, the median (IQR) year of genotyping was 2010 (2009–12) and the proportion of sequences from subtype B-infected patients was 82.7%.

Fifty-four out of 156 (34.6%) sequences carried at least one primary raltegravir resistance mutation. Raltegravir resistance prevalence is reported in Table 3. N155H was the most prevalent primary resistance mutation observed (16.0%), followed by Q148H/K/R (10.9%) and Y143C/H/R (9.6%). Thirteen (8.3%) sequences showed only secondary resistance-associated mutations (such as T97A, V151I, G163R and D232N); 3 (23.1%) of these 13 sequences were present at a viraemia level of 51–500 copies/mL (Table 3).

The prevalence of raltegravir resistance varied according to viraemia level (Table 3). Interestingly, raltegravir primary resistance was detected even at LLV. In particular, this prevalence was 18.2% and 37.5% at viraemia levels of 51–500 and 501–1000 copies/mL, respectively.

An equal prevalence of Q148H/K/R (H=1, K=0 and R=2) and N155H was found at LLV (viraemia 51–500 copies/mL: 9.1%; viraemia 501–1000 copies/mL: 18.8%), while Y143C/H/R was absent at LLV.

At a viraemia level of 1001–10000 copies/mL, the highest prevalence of raltegravir resistance (53.7%) was found. At this viraemia level, N155H was the most prevalent mutation (22.0%), followed by Y143C/H/R (17.1%; C/H/R=1, C=2 and R=4) and Q148H/K/R (14.6%; H=4, K=0 and R=1).

At viraemia levels >10000 copies/mL, the resistance prevalence was ~30% (Table 3). Interestingly, at these levels, three sequences carried both mutations Y143C/H/R and N155H.

### Evaluation of INSTI genotypic susceptibility in patients failing raltegravir

Among the overall 156 sequences analysed, 57.1%, 6.5% and 36.4% showed susceptible, intermediate resistant and resistant raltegravir GSS, respectively. As expected, by stratifying GSS according to viraemia level, a high concordance was found between the proportion of sequences with GSS equal to zero



**Table 3.** Resistance to raltegravir stratified for plasma viraemia range

Viraemia range (copies/mL)	n	Samples with at least one primary raltegravir mutation		Samples with Y143C/H/R		Samples with Q148H/K/R		Samples with N155H		Samples with only secondary resistance mutations	
		n (%)	P <sup>a</sup>	n (%)	P <sup>a</sup>	n (%)	P <sup>a</sup>	n (%)	P <sup>a</sup>	n (%)	P <sup>a</sup>
Overall range	156	54 (34.6)	0.421	15 (9.6)	0.028	17 (10.9)	0.561	25 (16.0)	0.702	13 (8.3)	0.860
51–500	33	6 (18.2)		0 (0.0)		3 (9.1)		3 (9.1)		3 (9.1)	
501–1000	16	6 (37.5)		0 (0.0)		3 (18.8)		3 (18.8)		0 (0.0)	
1001–10000	41	22 (53.7)		7 (17.1)		6 (14.6)		9 (22.0)		4 (9.8)	
10001–100000	40	12 (30.0)		4 <sup>b</sup> (10.0)		3 (7.5)		7 <sup>b</sup> (17.5)		6 (15.0)	
>100000	26	8 (30.8)		4 <sup>c</sup> (15.4)		2 (7.7)		3 <sup>c</sup> (11.5)		0 (0.0)	

<sup>a</sup>Potential differences in the percentage of resistance among the different viraemia ranges were evaluated by the  $\chi^2$  test for trend. *P* values <0.05 were considered as statistically significant.

<sup>b</sup>Two samples showed N155H+Y143C/H/R (C/H/R=1 and C=1).

<sup>c</sup>One sample showed N155H+Y143C.

(Figure 1a) and the proportion of sequences with at least one primary raltegravir resistance mutation (Table 3). A low proportion of samples showed intermediate resistant GSS (Figure 1a). These samples carried only accessory raltegravir resistance mutations such as T97A, G163R and V151I, whose role in raltegravir susceptibility should be clarified.

Cross-resistance to elvitegravir was found in 28.3% of the overall sequences; the highest prevalence of resistance was found at the 1001–10000 copies/mL stratum, while lower prevalence values were found at lower and higher viraemia strata (Figure 1b). Interestingly, a consistent intermediate resistant elvitegravir GSS (mainly due to the presence of Y143C/H/R) was found at viraemia >1000 copies/mL.

Regarding the second-generation INSTI dolutegravir, the proportion of sequences with resistance was very low (intermediate resistance: 14.7%; high resistance: 1.1%). No significant differences in the proportion of resistance levels according to viraemia ranks was observed (Figure 1 panel C).

The only two sequences with considerable resistance to dolutegravir (both at viraemia levels >1000 copies/mL) showed mutations Q148K+G140A+E138K and Q148H+G140S+G163G/R, respectively.

### Evaluation of the emergence of INSTI resistance according to the duration of raltegravir treatment

By stratifying samples according to the time of raltegravir treatment, no significant difference in terms of primary resistance prevalence was observed [treatment duration in months, *n* (%): <3, 12 (40.0); 3–6, 9 (32.1); 6–9, 9 (42.9); 9–12, 7 (36.8); and >12, 29 (34.5); *P*=0.703]. By analysing sequences at early failure (<3 months of raltegravir treatment, *n*=30), 12 (40.0%) sequences carried at least one primary raltegravir resistance mutation; of these, 5 had a viral load <1000 copies/mL (Figure 2). N155H was the most prevalent primary raltegravir mutation, found in six sequences (three of them with viraemia <1000 copies/mL). Mutations at position Q148 were found in four sequences (two of them with viraemia <1000 copies/mL). Thus, the primary

raltegravir resistance mutations Q148H/R and N155H can be found starting from the first 3 months of raltegravir treatment and even at LLV.

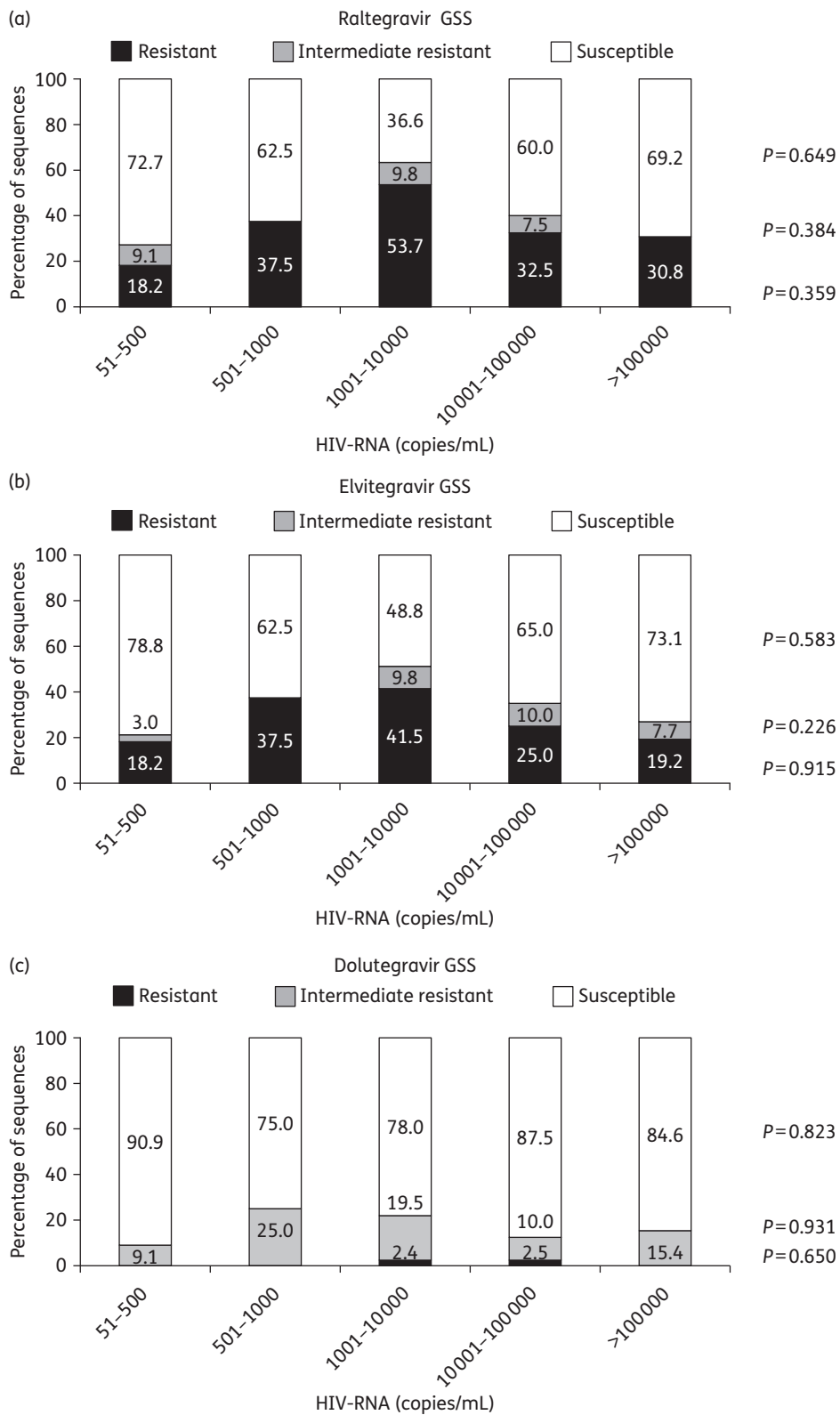
Two sequences showed Y143R mutation (both with a viral load >1000 copies/mL). The remaining 17 (56.7%) sequences did not show any integrase resistance-associated mutation at early failure on raltegravir.

## Discussion

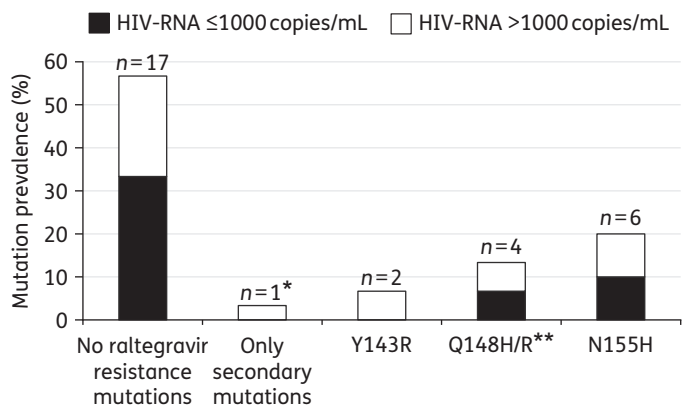
This study aimed at evaluating the integrase genotyping success rate even at LLV in a large dataset of samples tested in two clinical centres in Italy. Our results showed that the genotyping success rate was 95.7% for the overall population. In particular, this success rate was very high also for viral load levels below ≤500 copies/mL (~82%), reaching 94% at 501–1000 copies/mL and >94% at >1000 copies/mL. In light of the increasing usage of INSTIs, recently approved for all categories of HIV-1 patients,<sup>11,15,22</sup> these results demonstrate that integrase resistance monitoring can be performed also at LLV.

In this regard, the ability to easily analyse samples with LLV is mainly due to the improvement of the amplification step performed in our laboratories. As previously described for protease/reverse transcriptase genotyping,<sup>26</sup> the usage of nested PCR (especially for samples with LLV) did not significantly affect either the cost/timing of genotyping or its reliability and reproducibility at different viraemia levels. Indeed, by analysing the sequences from 41 plasma samples processed in duplicate in the two reference centres, a tight relatedness and an identical resistance profile between sequences from the same sample were found even at viraemia levels ≤1000 copies/mL. Moreover, by evaluating 233 integrase sequences obtained from 85 patients with at least two GRTs performed at different times and with a contextual viraemia ≤1000 copies/mL and >1000 copies/mL, a very high homology among sequences from the same patient was observed (97.6%).

Our results provide new details about the performance of integrase genotyping since few data are currently available about this issue. To *et al.*<sup>27</sup> compared integrase genotyping between



**Figure 1.** INSTI genotypic susceptibility scores (GSS) according to viraemia level in samples from patients failing a raltegravir-containing regimen. White, grey and black bars represent the proportion of sequences with susceptible, intermediate and resistant GSS according to the HIVDB algorithm (<http://sierra2.stanford.edu/sierra/servlet/JSierra?action=algSequenceInput>), respectively. (a) Scores for raltegravir. (b) Scores for elvitegravir. (c) Scores for dolutegravir.



**Figure 2.** Resistance to INSTIs at early failure. Analysis performed on 30 sequences at early failure (within 3 months of raltegravir treatment). \*T97A+G163R. \*\*Q148R: three sequences (two of them with G140S); Q148H: one sequence (+G140S).

in-house and commercial assays, to illustrate integrase polymorphisms in HIV-1 B and non-B subtypes but only in samples with a viral load >1000 copies/mL. In another study, integrase genotyping was performed in patients failing raltegravir with detectable viral loads >20 copies/mL and raltegravir resistance was evaluated according to viraemia level. Although integrase resistance was detected even at <200 copies/mL, the performance and reproducibility of integrase genotyping according to viraemia level was not mentioned.<sup>18</sup>

Regarding the evaluation of resistance at raltegravir failure, in our population the prevalence of raltegravir resistance (defined as the presence of at least one primary resistance mutation among Y143C/H/R, Q148H/K/R and N155H) was 34.6%, similar to that found in the study by Malet *et al.*,<sup>18</sup> performed on 161 patients failing on raltegravir (28.6%). In contrast, in other studies based on data from clinical practice, the prevalence of integrase resistance was higher, ranging from 42.8% to 68%.<sup>39–42</sup>

This discrepancy in the prevalence of raltegravir resistance can probably be explained by heterogeneity in the choice of mutations considered for the resistance definition, the population analysed and, most importantly, the time of integrase genotyping during failure and the viraemia level at GRT.

In our study, the prevalence of raltegravir resistance varied according to viraemia level. In particular, the highest prevalence of resistance was observed at viraemia levels of 1001–10 000 copies/mL, while resistance was lower at lower and higher viraemia strata. Similar results were observed by Malet *et al.*,<sup>18</sup> even though the viraemia stratification used was different. The decline in the prevalence of resistance observed at the very high viral load strata might reflect suboptimal medication adherence, with lower drug resistance selection.

We found that at LLV the prevalence of resistance ranged between 18.2% and 37.5%, with the presence of Q148H/K/R and N155H having similar prevalence (9.1% at a viraemia level of 51–500 copies/mL; 18.8% at viraemia 501–1000 copies/mL). Mutations C/H/R at position Y143 were not detected at viraemia <1000 copies/mL, underlining their association with high viral load.

Our GSS data support the hypothesis that cross-resistance between raltegravir and elvitegravir is also evident at LLV. On the other hand, resistance to dolutegravir was observed only in

two sequences (1.1%), both at viraemia >1000 copies/mL, carrying Q148H/K plus two accessory mutations (G140A+E138K and G140S+G163G/R). This mutational pathway is known to confer reduced susceptibility to dolutegravir.<sup>9,43</sup>

Analysing the association between the raltegravir primary resistance and the duration of raltegravir treatment, we found that Q148H/R (alone or together with the accessory mutation G140S) and N155H are detected during the first 3 months of raltegravir treatment and even at LLV. This finding suggests the importance of careful monitoring of resistance at early timepoints and/or LLV in order to avoid an accumulation of resistance.<sup>29</sup> It should be highlighted that the maintenance of raltegravir pressure on viruses carrying Q148H/K/R or N155H has been associated with an accumulation of accessory resistance mutations (which increase the phenotypic resistance and viral fitness of resistant viruses) or with the switch of N155H to Q148H/K/R mutation.<sup>16,44–46</sup>

This study might have some limitations. First, the majority of the 1734 samples analysed were genotyped from patients never exposed to INSTI selective pressure. Indeed, even though these samples were useful to evaluate the performance of integrase genotyping, the reliability of the assay to detect resistance mutations was tested in a subgroup of 182 samples. This limitation is mainly due to the fact that at the moment the number of raltegravir failures in clinical practice is very low because of both the high rates of virological success in raltegravir-treated patients and the recent introduction of INSTIs in the clinic. Further analyses with a higher number of samples are warranted to confirm these data.

A second limitation could be that in our analyses, despite the overall high success rate, the success rate in samples from patients infected by non-B subtypes was significantly lower than in those infected by subtype B (91% versus 96%). This discrepancy was mainly observed in samples with recombinant forms.

Finally, the resistance at LLV was only quantitatively described, without any evaluation of its impact on subsequent virological outcome. This point was outside the primary scope of this article, yet an analysis was performed on a subset of 18 patients in first-line regimen for whom a GRT was requested at viraemia levels of 51–1000 copies/mL. Even though this analysis was performed on a very small population, the probability of reaching viraemia >1000 copies/mL by 1 year after LLV was significantly higher in patients with at least one primary raltegravir resistance mutation than in those without resistance (100% versus 14.9%;  $P=0.011$ ; data not shown). This suggests that early detection of resistance (when viraemia is still  $\leq 1000$  copies/mL) may prevent evolution towards (i) virological failure with higher viraemia and (ii) the accumulation of additional mutations, thus affecting the choice of future therapeutic regimens, as previously proposed.<sup>30,47</sup>

In conclusion, the INSTI class, with three drugs suitable for both drug-naïve and multi-experienced HIV-1-infected patients, is an exceptional opportunity for modern cART to ensure greater chances of virological success. However, this opportunity should only be grasped in combination with resistance monitoring. The reliability and reproducibility of HIV-1 integrase GRT guarantees the detection of primary resistance mutations either at early timepoints or at LLV. These findings may be useful in the management of failures even at LLV to avoid the accumulation of mutations and cross-resistance within the INSTI class.

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## Supplementary data

Supplementary Methods, Table S1 and Figures S1–S3 are available as Supplementary data at JAC Online (<http://jac.oxfordjournals.org/>).

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