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Evaluation of HIV-1 integrase resistance emergence and evolution in patients treated with integrase inhibitors

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Highlights

- In INSTI-naïve patients, major INSTI-resistance mutations occur very rarely
- In INSTI treated patients, N155H is the most common major mutation selected
- The emergence of resistance is related with a higher integrase evolution

Abstract

Objectives: We evaluated the emergence of mutations associated to integrase strand transfer inhibitors (INSTI) resistance (INSTI-RMs) and the integrase evolution in HIV-1 infected patients treated with this drug class.

Methods: Emergence of INSTI-RMs and integrase evolution (estimated as genetic distance between integrase sequences under-INSTI and before-INSTI treatment) were evaluated in 107 INSTI-naïve patients (19 drug-naïve and 88 drug-experienced) with two plasma genotypic resistance tests available: one before and one under INSTI treatment. A logistic regression analysis was performed to evaluate factors associated with the integrase evolution under INSTI treatment.

Results: Patients were mainly infected by B subtype (72.0%). 87 patients were treated with raltegravir, 13 with dolutegravir and 7 with elvitegravir. Before INSTI treatment, one patient harboured the major INSTI-RM R263K, and three patients the accessory INSTI-RMs T97A. Under INSTI treatment, the emergence of ≥1 INSTI-RM was found in 39 (36.4%) patients. The major INSTI-RMs which emerged more frequently were: N155H (17.8%), G140S (8.4%), Y143R (7.5%), Q148H (6.5%), Y143C (4.7%).

Concerning integrase evolution, a higher genetic distance was found in patients with ≥1 INSTI-RM compared to those without emergence of resistance (0.024 [0.012-0.036] vs. 0.015 [0.009-0.024], p=0.018). This higher integrase evolution was significantly associated with a longer duration of HIV-1 infection, a higher number of past regimens and non-B subtypes.

Conclusions: Our findings confirmed that in INSTI-naïve patients, major INSTI-RMs occur very rarely. Under INSTI treatment, selection of drug-resistance follows the typical drug-resistance pathways; a higher evolution characterizes integrase sequences developing drug-resistance compared to those without any resistance.

Keywords: HIV-1 integrase resistance; genetic distance; integrase inhibitors; polymorphisms; subtype.

Introduction

Integrase strand transfer inhibitors (INSTIs) are potent inhibitors of the HIV integrase enzyme, targeting the strand transfer reaction required to incorporate viral DNA into the host genome [1]. INSTIs are recommended both in first-line and in rescue therapy because of their high potency and improved tolerability in both naïve and treatment-experienced patients [2]. INSTI-resistance mutations located in the integrase gene and selected by INSTI exposure have been described *in vitro* and *in vivo* [3]. In clinical trials, dolutegravir (DTG) and bictegravir (BIC), the most recently approved second-generation INSTIs, have shown a higher genetic barrier with respect to the first-generation INSTIs, raltegravir (RAL) and elvitegravir (EVG). Indeed, INSTI-resistance mutations emerge frequently during RAL and EVG treatment when combined with other antiretroviral drugs, and a single amino acid substitution in the integrase region is sufficient to confer high-level resistance to these drugs. In particular, N155H or Q148R/H/K confers high-level cross-resistance to RAL and EVG, while other pathways lead to more specific resistance to EVG (T66I or E92Q/G)

and to RAL (Y143R/H/C). By contrast, emerging resistance to DTG, including the novel R263K mutation, has been reported during DTG monotherapy [4] and anecdotally in INSTI naïve antiretroviral experienced patients [5,6]. However, it should not be underestimated that DTG activity is also significantly diminished in the presence of the Q148R/H/K pathway, particularly when additional mutations such as G140S/A and E138K are present [7]. While BIC has not yet been used in patients with previous INSTI failure, *in vitro* data suggest that BIC and DTG resistance profiles overlap extensively [8].

Potential issues with interpretation of genotypic INSTI-resistance include the limited clinical experience outside HIV-1 subtype B [9], the occurrence of poorly defined alternative resistance pathways [10-13] and the plethora of integrase polymorphisms and minor mutations modulating INSTI-resistance, some of which are subtype specific [14-16]. Indeed, the most widely used genotype interpretation systems include different sets of accessory mutations and assign different weights to rare mutations, possibly resulting in divergent indications [17-18]. Genotype-treatment correlation data is a major source for investigating the role of HIV mutations. For example, the Stanford HIVdb site allows the retrieval of HIV integrase genotype information from INSTI-naïve versus INSTI-treated patients providing data for cross-sectional analysis of the prevalence of integrase mutations in the two datasets. However, in principle the most informative data should be derived from paired intrapatient analysis of pre- versus post-INSTI treatment HIV genotype, allowing direct analysis of HIV evolution under INSTI pressure. The aim of this study was to analyze the emergence of integrase mutations and the consequent integrase evolution in a population of INSTI-treated patients attending routine care in several Italian clinical centers, with available plasma integrase sequences before and after starting INSTI treatment.

2. Materials and methods

2.1. Study population

One-hundred and seven (19 drug-naïve and 88 drug-experienced) patients, starting for the first time a regimen containing INSTIs, were selected for this analysis. Eligible individuals were those for whom two plasma integrase genotypic resistance tests (GRTs) were available, one before and one under INSTI treatment. Integrase genotype sequences from the individuals selected for the study were retrieved from the Antiviral Response Cohort Analysis (ARCA) database (https://www.dbarca.net/) and from a large Italian anonymous database which collects data from for HIV-1 infected patients followed at several clinical centres in Central Italy. Data collection was approved by the local Ethics Committees, and written informed consent was obtained from all patients before participation. The study was performed in accordance with the ethical principles of the Declaration of Helsinki (7th revision) and with the International Conference on Harmonization Good Clinical Practice guidelines. All integrase sequences (including the first 263 amino acid positions) were obtained from plasma samples by Sanger's population sequencing using commercially available or homebrew systems, as previously described [19].

2.2. Analysis of HIV-1 Integrase sequences

Amino acid frequencies across all integrase codons were evaluated in GRTs obtained before and under INSTI treatment. The emergence of major and accessory INSTI-resistance mutations (INSTI-RMs), reported in Stanford HIV Drug Resistance Database list 2018 (https://hivdb.stanford.edu/dr-summary/resistance-notes/INSTI/) were evaluated.

2.3. Covariation analysis

To evaluate potential associations between integrase mutations before INSTI treatment and the emergence of INSTI-resistance mutations under INSTI treatment a covariation analysis was performed. For this analysis, we considered all integrase mutations present before INSTI treatment with a prevalence >3% and all INSTI-resistance mutations under INSTI treatment. We calculated the phi coefficient for all the possible pairwise combinations by using a script implemented in the R

software, version 3.4.1. Statistically significant pairwise associations were those with a P value < 0.05.

2.4. Analysis of HIV-1 integrase evolution

In order to define the integrase evolution during INSTI treatment, the degree of genetic divergence between the integrase sequences under and before INSTI treatment was calculated. In particular, a pairwise genetic distance (based on Tajima Nei model, Mega6 [20]) was obtained for each pair of integrase sequences, stripped at positions related to INSTI-resistance mutations (both major and accessory).

2.5. Statistical analyses

McNemar's test was used to compare amino acid frequencies across all integrase codons in integrase sequences obtained before and under INI-treatment.

Fisher's exact and Pearson Chi-squared test (for categorical variables) and Mann–Whitney and Kruskal-Wallis test (for quantitative variables) were used to define statistically significant differences between patients with or without at least one INSTI-resistance mutations compared to those without emergence of resistance and between different INSTI types. All P-values for multiple pairways comparisons were adjusted by using Benjamini and Hochberg correction [21].

Multivariable logistic regression analysis was also performed in order to evaluate factors independently associated with integrase evolution under INSTI treatment, by adjusting for the following variables: HIV-1 subtype, years of HIV- infection, number of previous regimens, integrase inhibitors drugs, duration of therapy at GRT under integrase inhibitor-treatment, time between the two GRTs and plasma viral load at GRT under INSTI treatment. For this analysis the integrase evolution was evaluated by considering the median value of genetic distance as cut-off (<0.018 versus >0.018).

All these analyses were performed using the statistical software package SPSS (version 19.0) for Windows (SPSS Inc., Chicago, IL, USA) and R open source environment for statistical computing (version 3.4.1).

3. Results

3.1. Patients' characteristics

Patients' characteristics of the overall population and according to INSTI used are reported in Table 1. The population was mainly composed by males (63.6%) and Italians (72.6%) with a median (interquartile, IQR) age of 42 (35-47) years. The majority of individuals were infected by HIV-1 subtype B (75.7%). Nineteen patients started an INSTI-based treatment as drug-naïve, while 88 were drug-experienced and started the INSTI-based regimen after failing therapy. Drug-experienced patients had a median (IQR) number of 6 (2-11) previous regimens. The median (IQR) year of starting an INSTI-based regimen was 2013 (2009-2015). The median (IQR) duration of this INSTI-therapy at the moment of GRT was 11.4 (6.5-25.5) months. The median (IQR) time between the two GRTs was 12.0 (7.9-28.2) months. The median (IQR) HIV-1 RNA at GRT before INSTI treatment was 4.7 (3.7-5.4) $\log_{10}$ copies/ml. Looking at the INSTI administered, 87 patients were treated with RAL, 13 with DTG and 7 with EVG. After therapy starting, 53 patients (49.5%: 9 [47.4%] drug-naïve and 44 [50%] drug-experienced) achieved virological undetectability (plasma HIV-RNA < 50 copies/mL); only five of them maintained the undetectability at second GRT.

3.2. Evaluation of resistance mutations

Integrase sequences were analysed before and under INSTI treatment for the 107 subjects included in the study. Considering the resistance at GRT performed before INSTI treatment, we observed that three patients harboured the accessory resistance mutation T97A, while one patient harboured the major INSTI-mutation R263K. Regarding the resistance at GRT performed under INSTI treatment, in 39 (36.4%) patients we observed the emergence of at least one INSTI-resistance mutation. All these 39 patients were under virological failure at the moment of GRT. The

emergence of INSTI-resistance mutations was found with a significant higher prevalence (almost double) in patients who never achieved virological suppression under INSTI-treatment compared to those who achieved undetectability and after failed (25/54 [46.3%] versus 14/53 [26.4%], p=0.044). Major INSTI-resistance mutations at GRT were found in a similar proportion of patients in those drug-naïve and drug-treated (7/19 [36.8%] versus 32/88 [36.4%], p value=1). The median (IQR) number of INSTI-resistance mutations observed in the population was 2 (1-2).

The most commonly observed major INSTI-resistance mutations that emerged under INSTI treatment with a prevalence >3% were N155H (19, 17.8%; p<0.001), G140S (9, 8.4%; p=0.004), Y143R (8, 7.5%; p=0.008), Q148H (7, 6.5%) and Y143C (5, 4.7%) (Figure 1).

Among the accessory INSTI-resistance mutations, the prevalence of T97A significantly increased under INI-treatment from 2.8% to 12.1% (p=0.002). The following accessory mutations emerged under INSTI treatment: L74M (1, 0.9%), E138T (1, 0.9%), E157Q (2, 1.9%) and S230R (3, 2.8%). The prevalence of all other accessory mutations did not significantly vary before and under INSTI treatment. No other differences between GRTs before and under INSTI treatment were observed in all other codons across all integrase region.

3.3. Evaluation of resistance mutations according to type of INSTI used and subtype

By evaluating the prevalence of major INSTI-resistance mutations according to type of INSTI used, the emergence of at least one INSTI major mutation was found in 34/87 (39.1%) patients treated with RAL. The most prevalent mutation was N155H (17.2%), followed by G140S (8.0%) and Y143R (8.0%) (Table 2). The most prevalent patterns of mutations were G140S+Q148H/R (6, 6.9%) and T97A+Y143C/H/R (5, 5.7%) (Supplementary Table 1).

The emergence of at least one major INSTI mutation was found in 3/13 patients treated with DTG (23.1%). The most prevalent mutations were N155H, G140S and Q148H, present in combination in two patients (15.4%) as follows: N155H+Q148H/R+G140S (N=1) and N155H+Q148H/R+G140S+E138A/K (N=1) (Table 2 and Supplementary Table 1).

N155H was the only major mutation found in the 7 patients treated with EVG (2, 28.6%) (Table 2). By considering the prevalence of major INSTI mutations according to subtype, N155H was the only mutation present in patients infected with HIV-1 non-B subtype (26.9% in non-B subtypes versus 14.8% in B subtypes; p=0.24).

3.3. Evaluation of integrase mutations before INSTI treatment associated with the emergence of INSTI-resistance mutations

A further step of our study was to analyse the potential associations between the presence of specific integrase mutations at baseline of INSTI treatment and the further emergence of INSTI-resistance mutations under INSTI treatment. (Table 3).

In patients infected by HIV-1 non-B subtypes (N=26), we found that three patients (11.5%) harboured the I113V mutation at baseline. This mutation was associated with the emergence of N155H (phi=0.59, p value=0.013) during INSTI treatment. In patients infected by B subtype (N=81), the presence of the D286N mutation at baseline was observed in six patients (7.4%); this mutation was associated with the emergence of both G140S (phi=0.50, p=0.001) and (even if less strongly) Q148H/R (phi=0.35, p=0.016) under INSTI treatment (Table 3).

3.4. Evaluation of integrase evolution

Concerning the integrase evolution, genetic distance between the integrase sequences performed before INSTI treatment and those performed under INSTI treatment was estimated excluding the codons associated with INSTI-resistance mutations (both major and accessory). The median (IQR) genetic distance observed in our population was 0.018 (0.009-0.028).

The genetic distance was significantly higher in integrases developing at least one INSTI-resistance mutation (median [IQR]: 0.024 [0.012-0.036]) compared to those who remained fully susceptible to INSTIs (0.015 [0.009-0.024]) (p=0.013) (Figure 2A). No differences in genetic distance were observed by stratifying for the different INSTIs used (p=0.462) (Figure 2B).

Factors independently associated with the integrase evolution under INSTI treatment were evaluated by multivariable logistic regression analysis. For this analysis the integrase evolution was evaluated by considering the median value of genetic distance as cut-off (<0.018 versus >0.018).

Factors positively associated with integrase evolution were a longer duration of HIV-1 infection (per 1 year increase; Adjusted odds ratio, AOR [95% Confidence interval, CI]: 1.14 [1.05-1.24], p=0.002), a lower number of antiretroviral regimens previously administered (per 1 regimen less; AOR [95% CI] 1.11 [1.00-1.23], p=0.045), and non-B versus B subtype (with a trend of significance: AOR [95% CI] 3.46 [0.97-12.36], p=0.056) (Table 4).

4. Discussion

INSTIs have become an essential element of modern antiretroviral treatment in both drug-naïve and experienced patients [2,22]. Given the increasing use of this class of antiretrovirals, the characterization of the integrase region, in terms of the emergence of integrase mutations and the consequent evolution of this region, could help to understand if clinically relevant mutations and polymorphisms occur and if their presence could influence the clinical response to INSTIs.

To answer these questions, in the present study we evaluated the prevalence of all amino acid positions of integrase in a population of patients who started a regimen containing an INSTI for the first time and attended routine care in several Italian clinical centers.

Analysing the HIV-1 integrase sequences obtained from 107 HIV-1 infected individuals before INSTI treatment, we found that the prevalence of major integrase mutations was very rare, confirming the data so far reported in the literature. In fact, studies about the presence of transmitted resistance to INSTIs showed a very low or absent presence of major integrase mutations [23-29]. In our study, before INSTI treatment, R263K was the only major mutation found in one patient before INSTI treatment; by contrast, several polymorphisms contributing to INSTI-resistance were found, as observed in other studies [23,24].

Under INSTI treatment, the overall prevalence of patients with emergent major INSTI-resistance mutations was 36.4%, clearly higher than that found at 48 weeks in randomized clinical trials [30-38], but this is normal because patients included in the trials are well selected patients, with good clinical and viro-immunological characteristics. By evaluating other findings from real clinical settings, our prevalence of emergent INSTI-resistance mutations was similar to that (39.6%) found by Nguyen and colleagues in their study on 134 patients failing an INSTI-based regimen (65 failed under RAL, 20 under EVG and 49 under DTG) [39]. Our results are also in line with other two studies evaluating the emergence of INSTI-resistance in patients failing a RAL-based regimen [9, 40].

In contrast, other recent studies showed a lower prevalence of INSTI-resistance in INSTI-experienced patients (from <1% to 11.7%-22%) [6,26,27]. This apparent discrepancy in the prevalence of INSTI-resistance can be explained by a different assessment of integrase mutations (as major or accessory) and the different patients' characteristics. Concerning this last point, for example, in the study by Lepik et al. [6], authors analysed only patients treated with two NRTIs plus one INI, while in our population only about 25% of patients were treated with this recommended drug combination (data not shown); the other patients were under alternative regimens (some of them including at least four drugs) because they were pluri-treated with a long and complicated history of treatment (data non shown). Moreover, in Lepik's study, around 50% of patients started the INSTI-regimen under virological suppression, while our patients were all with baseline plasma HIV-RNA above 50 copies/mL (because drug-naïve or under virological failure).

In the present study, all patients with emergent INSTI-resistance mutations were under virological failure. Interestingly, a significant higher percentage of patients who never achieved virological suppression harboured INSTI-resistance compared to those who achieved virological suppression and after failed (46.3% versus 26.4%), confirming that these last ones are less prone to accumulate drug-resistance. As expected, major INSTI-resistance mutations at GRT under INSTI-treatment were found with a similar proportion in drug-naïve and drug-experienced patients (36.8% versus

36.4%). In fact, even though drug-experienced patients were more fragile because their longer time of infection and their previous experience to drugs, all these patients (both drug-naïve and drug-experienced) did never have an experience with INSTIs before. In our study, the selection of drug-resistance followed the typical drug-resistance pathways. In particular, the most widespread major mutations found under INSTI treatment were N155H (17.8%), followed by G140S (8.4%), Y143R (7.5%) and Q148H (6.5%). In line with this result, the recent paper by Nguyen showed that the most prevalent pathways of resistance were N155H (45.2%) and Q148H/K/R (22.6%). In our study, the N155H, besides being the most prevalent mutation in the overall population, was the mutation present indistinctly from the type of INSTI used. Furthermore, this was the only mutation present in patients infected with a non-B subtype.

Regarding the accessory INSTI-resistance mutations, the prevalence of T97A significantly increased under INSTI treatment (p=0.002), while the prevalence of all other accessory mutations did not significantly change before and under INSTI treatment. By analysing all other codons across integrase, no other differences were observed between GRTs before and under INSTI treatment.

In the present study, we found that the presence of INSTI-resistance in INSTI-treated patients maybe related to specific integrase mutations at baseline of INSTI treatment, potentially promoting the selection of major INSTI-resistance mutations. In particular, patients infected with HIV-1 viruses carrying D286N (for B subtypes) and I113V (for non-B subtypes) harbour the INSTI-mutations G140S, G148H/R, and N155H more frequently than those infected by viruses wild type at INI positions 286 and 113 (Table 3). However, as these two “novel” mutations were found in a very small number of patients, our results need to be confirmed in a larger population. Moreover, further studies based on refined structural analyses and docking simulations are needed to confirm these findings.

We also aimed at evaluating integrase evolution under INSTI-pressure by the estimation of genetic distance. A higher integrase evolution from baseline to follow-up was found in sequences developing INSTI-resistance compared to those not developing resistance. When a multivariable

model was applied to define factors significantly associated with a higher genetic distance, a longer duration of HIV-1 infection, a higher number of antiretroviral regimens and non-B subtypes were all factors associated with a greater genetic distance from baseline to follow-up.

The association between higher integrase evolution and non-B subtypes could be explained by the fact that patients infected with these non-B subtypes had a longer history of HIV infection and had a higher number of antiretroviral treatments compared to other patients.

Our analysis has a number of limitations. Firstly, the study was performed on a small number of patients therefore we could not get to appreciate potential differences between subtype B and non-B subtypes. However, the favourable point is the availability of integrase before and under INSTI treatment for each patient. Secondly, the number of patients treated with EVG and DTG is small compared to those treated with RAL. More studies in a larger cohort of patients infected by non-B subtypes and treated with DTG and EVG are needed. Furthermore, data on BIC are missing, due to the very recent introduction of this INSTI in clinical practice.

5. Conclusions

In conclusion, this study provides data for the clinical practice and treatment with INSTIs. Our findings confirm that the prevalence of mutations associated with resistance to this antiretroviral class in INSTI-naïve patients is still very rare. However, under INSTI treatment the selection of INSTI-resistance mutations occurs (regardless the type of INSTI used), and follows the typical INSTI-resistance pathways. The presence of two integrase mutations I113V and D286N at baseline seem to be involved in the selection of mutations associated with resistance, but this finding needs to be investigated more deeply. Samples developing INSTI-resistance under treatment are characterized by a higher integrase evolution.

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Declarations

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Legends to figures

Figure 1. Frequency of major and accessory INSTI-resistance mutations before and under INSTI treatment. The major INSTI-resistance mutations T66A/I/K, G118R, G140A/C, Q148K and the accessory mutations Y143K/S/G/A, P145S, Q146P, S147G, Q148N, G149R, V151I/L/A, S153Y/F, N155S/T/D did not co-occur in our study. After correction for multiple comparisons, N155H was the only mutation that maintained a statistically significant difference in the prevalence before and under INSTI treatment. P-values were calculated by McNemar's test.

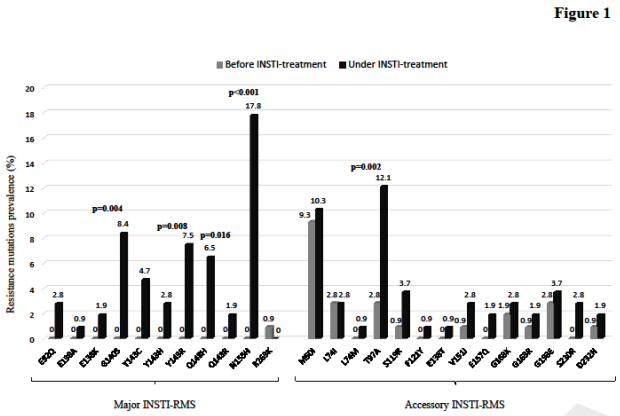
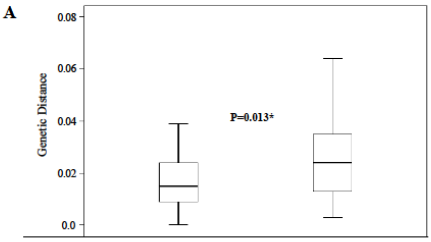


Figure 2. Distribution of HIV-1 integrase genetic distances: (A) Distribution of HIV-1 integrase genetic distances, stratified for patients without INSTI-resistance mutations and patients with at least one INSTI-resistance mutation. **(B)** Distribution of HIV-1 integrase genetic distances according to INSTI drugs. *Statistically significant differences were assessed by Mann Whitney or Kruskal-Wallis tests, as appropriate. RM: resistance mutation. Pts: patients.

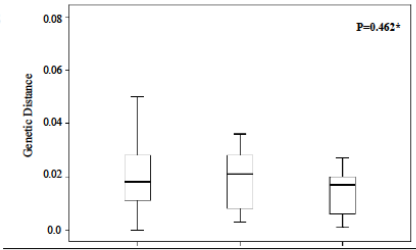
Figure 2

A



Genetic Distance	Pts without INSTI-RMs (N=68)	Pts with ≥1 INSTI-RM (N=39)
Median, IQR	0.015 (0.009-0.024)	0.024 (0.012-0.036)
Mean±SE	0.018±0.015	0.026±0.016

B



Genetic Distance	RAL (N=87)	DTG (N=13)	EVG (N=7)
Median, IQR	0.018 (0.01-0.028)	0.021 (0.008-0.028)	0.017 (0.005-0.021)
Mean±SE	0.022±0.016	0.022±0.016	0.014±0.01

Table 1. Patients' characteristics.

[a] Other subtypes in the overall population were: A1 (N=1, 0.9%); C (N=2, 1.9%); D (N=1, 0.9%); G (N=2, 1.9%); CRF01_AE (N=2, 1.9%); CRF12_BF (N=2, 1.9%); CRF40_BF

Variables	Overall, N=107	INSTI received				Therapeutic status		
		RAL, N=87	DTG, N=13	EVG, N=7	P-value[b]	Drug-naïve, N=19	Drug-experienced, N=88	P-value[c]
Male, N (%)	68 (63.6)	56 (64.4)	8 (61.5)	4 (57.1)	0.779	14 (73.7)	54 (61.4)	0.522
Italians, N (%), (N=106)	77 (72.6)	61 (70.1)	11 (91.7)	5 (71.4)	0.291	13 (68.4)	64 (72.7)	0.421
Age, years, median (IQR), (N=99)	42 (35-47)	42 (36-49)	35 (28-41)	42 (34-48)	0.065	44 (34-48)	42 (36-46)	0.493
Drug-naive, N (%)	19 (17.8)	14 (16.1)	2 (15.4)	3 (42.9)	0.198	-	-	-
Subtype, N (%):								
B	81 (75.7)	67 (77.0)	11 (84.6)	3 (42.9)	0.101	10 (52.6)	71 (80.7)	**0.017**
CRF02_AG	7 (6.5)	6 (7.0)	1 (7.7)	0 (0.0)	0.999	1 (5.2)	6 (6.8)	1.000
F1	8 (7.5)	7 (8.0)	0 (0.0)	1 (14.2)	0.446	4 (21.1)	4 (4.5)	**0.032**
Other [a]	11 (10.3)	7 (8.0)	1 (7.7)	3 (42.9)	**0.036**	4 (21.1)	7 (8.0)	0.104
Year of first seropositivity, median (IQR) (N=98)	1997 (1990-2007)	1997 (1990-2006)	1995 (1989-2000)	2009 (2002-2014)	0.256	2014 (2009-2015)	1996 (1989-2002)	**<0.001**
Year of first-line regimen, median (IQR)	1999 (1995-2009)	1999 (1995-2009)	2002 (1995-2009)	2009 (2003-2014)	0.125	2014 (2011-2015)	1998 (1995-2006)	**<0.001**
Number of previous regimens, median (IQR)	5 (1-10)	5 (1-17)	8 (2-13)	2 (0-11)	0.297	0 (0-0)	6 (2-11)	**<0.001**
Year of starting INSTI treatment, median (IQR)	2013 (2009-2015)	2011 (2008-2014)	2016 (2015-2016)	2015 (2014-2015)	**<0.001**	2014 (2011-2015)	2012 (2008-2014)	**0.006**
Duration of INSTI therapy, months, median (IQR)	11.4 (6.5-25.5)	11.0 (6.5-22.7)	9.2 (6.9-12.0)	16.1 (6.2-24.2)	0.462	9.6 (5.5-13.5)	11.6 (7.0-24.2)	0.061
Time between two GRTs, months. median (IQR)	12.0 (7.9-28.2)	11.2 (7.7-32.1)	15.3 (15.3-15.3)	19.4 (10.8-24.2)	0.665	13.3 (10.8-26.5)	13.8 (8.4-34.8)	0.628
HIV-1 RNA at GRT before INSTI treatment (log_{10} copies/ml), median (IQR)	4.7 (3.7-5.4)	4.6 (3.7-5.4)	4.9 (3.9-5.2)	5.8 (2.8-6.4)	0.562	5.7 (5.1-6.2)	4.4 (3.6-5.2)	**<0.001**
HIV-1 RNA at GRT under INSTI treatment (log_{10} copies/ml), median (IQR)	3.5 (2.5-4.4)	3.4 (2.5-4.2)	3.8 (3.3-5.0)	4.2 (2.2-5.1)	0.222	2.3 (1.8-3.2)	3.6 (2.7-4.7)	**<0.001**
Occurrence of ≥1 Major integrase RAM at GRT before INSTI treatment[d], N (%)	1 (0.9)	0 (0.0)	1 (7.7)	0 (0.0)	0.186	0 (0.0)	1 (1.1)	1.000
Occurrence of ≥1 Major integrase RAM at GRT under INSTI treatment, N (%)	39 (36.4)	34 (39.1)	3 (23.1)	2 (28.6)	0.586	7 (36.8)	32 (36.4)	1.000

(N=1, 0.9%). [b] Statistically significant differences between RAL, DTG and EVG were assessed by Kruskal-Wallis test and Pearson Chi-squared test (table 2X3), as appropriate. [c] Statistically significant differences between drug-naïve and drug-experienced patients were assessed by Mann-Whitney test and Chi-squared test (table 2X2), as appropriate. [d] only one major INSTI-resistance mutation was found before INSTI-treatment: The R263K mutation.
RAL: raltegravir; DTG: dolutegravir; EVG: elvitegravir; GRT: genotypic resistance test; IDU: injection drug user; INSTI: Integrase strand transfer inhibitors. IQR: interquartile.

Table 2. Prevalence of major integrase resistance mutations detected under treatment with integrase inhibitors according to drug and subtype.

MAJOR MUTATIONS	DRUGS			SUBTYPES	
	RAL (N=87) n (%)	DTG (N=13) n (%)	EVG (N=7) n (%)	B (N=81) n (%)	Non-B (N=26) n (%)
N155H	15 (17.2)	2 (15.4)	2 (28.6)	12 (14.8)	7 (26.9)[a]
G140S	7 (8.0)	2 (15.4)	0 (0.0)	9 (11.1)	0 (0.0)
Y143R	7 (8.0)	1 (7.7)	0 (0.0)	8 (9.9)	0 (0.0)
Q148H	5 (5.7)	2 (15.4)	0 (0.0)	7 (8.6)	0 (0.0)
Y143C	4 (4.6)	1 (7.7)	0 (0.0)	5 (6.2)	0 (0.0)
E92Q	3 (3.4)	0 (0.0)	0 (0.0)	3 (3.7)	0 (0.0)
Y143H	2 (2.3)	1 (7.7)	0 (0.0)	3 (3.7)	0 (0.0)
Q148R	2 (2.3)	0 (0.0)	0 (0.0)	2 (2.5)	0 (0.0)
E138K	2 (2.3)	0 (0.0)	0 (0.0)	2 (2.5)	0 (0.0)
E138A	0 (0.0)	1 (7.7)	0 (0.0)	1 (1.2)	0 (0.0)

[a] Subtypes: A1 (N=1), C (N=2), D (N=1), F1 (N=1), G (N=1), CRF02_AG (N=1).
DTG: dolutegravir; EVG: elvitegravir; RAL: raltegravir.

Table 3. Significant correlations between integrase mutations detected at first integrase genotypic test and INSTI resistance mutations detected under INSTI regimen

Integrase amino acid mutations at first integrase genotypic test[a]	Prevalence, n (%)	INSTI resistance mutations[b]	Prevalence, n (%)	Covariation frequency, n (%)[c]	Covariation frequency, n (%)[d]	Phi[e]	P-value[f]
Non-B subtypes (N=26)							

I113V	3 (11.5)	N155H	7 (26.9)	3 (100.0)	3 (42.9)	0.59	0.013
B subtypes (N=81)							
D286N	6 (7.4)	G140S	9 (11.1)	4 (66.7)	4 (44.4)	0.50	0.001
		Q148H/R	9 (11.1)	3 (50.0)	3 (33.3)	0.35	0.016

[a] Integrase amino acid mutation detected at first integrase genotypic test.
[b] INSTI drug resistance mutation detected at second integrase genotypic test.
[c] Covariation frequency based on the prevalence of integrase amino acid mutations detected at the first integrase genotypic test.
[d] Covariation frequency based on the prevalence of INSTI resistance mutations detected at the second integrase genotypic test.
[e] Positive correlations and negative correlations with phi >0.10 and phi <-0.10 are shown, respectively.
[f] All P values for covariation were significant at a false discovery rate of 0.05

Table 4. Multivariable logistic regression analysis of factors associated with integrase evolution[a].

Variables	**AOR (95%CI)**	**P-Value[b]**
Subtype		
B[c]	1	
Non-B	3.46 (0.97-12.36)	0.056
Years of HIV-1 infection (per 1 year increase)	1.14 (1.05-1.24)	**0.002**
Number of previous regimens (per 1 regimen less)	1.11 (1.00-1.23)	**0.045**
Integrase inhibitor		
Raltegravir[c]	1	
Dolutegravir	1.16 (0.24-5.54)	0.855
Elvitegravir	0.83 (0.12-5.61)	0.846
Time between the two GRTs (per 1 month increase)	1.01 (0.99-1.03)	0.103
Duration of therapy at GRT under INSTI-treatment (per 1 month increase)	0.99 (0.97-1.02)	0.867
HIV-1 RNA at GRT under INI-treatment (per 1 log_{10} increase)	1.04 (0.71-1.51)	0.855

[a] Integrase evolution was evaluated by considering the median value of genetic distance as cut-off (<0.018 vs. >0.018).

[b] Statistically significant p-value (<0.05) by multivariable logistic regression analysis are reported in bold.

[c] Reference group.

AOR: Adjusted odds ratio; CI: Confidence interval; GRT: genotypic resistance test; INSTI: Integrase strand transfer inhibitors