HIV integrase variability and genetic barrier in antiretroviral naïve and experienced patients

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Abstract

Background: HIV-1 integrase (IN) variability in treatment naïve patients with different HIV-1 subtypes is a major issue. In fact, the effect of previous exposure to antiretrovirals other than IN inhibitors (INI) on IN variability has not been satisfactorily defined. In addition, the genetic barrier for specific INI resistance mutations remains to be calculated.

Methods: IN variability was analyzed and compared with reverse transcriptase (RT) and protease (PR) variability in 41 treatment naïve and 54 RT inhibitor (RTI) and protease inhibitor (PRI) experienced patients from subjects infected with subtype B and non-B strains. In addition, four HIV-2 strains were analyzed in parallel. Frequency and distribution of IN mutations were compared between HAART-naïve and RTI/PI-experienced patients; the genetic barrier for 27 amino acid positions related to INI susceptibility was calculated as well.

Results: Primary mutations associated with resistance to INI were not detected in patients not previously treated with this class of drug. However, some secondary mutations which have been shown to contribute to INI resistance were found. Only limited differences in codon usage distribution between patient groups were found. HIV-2 strains from INI naïve patients showed the presence of both primary and secondary resistance mutations.

Conclusion: Exposure to antivirals other than INI does not seem to significantly influence the emergence of mutations implicated in INI resistance. HIV-2 strain might have reduced susceptibility to INI.

Background

Raltegravir (MK-0518; Isentress, Merck) was the first integrase (IN) inhibitor approved for treatment of HIV infection [1], while other compounds such as GS-9137 [2], S-1360 [3], and L-870,810 [4] are at different stages of development. Raltegravir (RAL) has shown potent and durable antiretroviral activity in both treatment naïve [5,6] and highly experienced HIV-1-infected individuals. Due to its novel mechanism of action, RAL was shown to be effective also against HIV-1 strains resistant to reverse transcriptase (RT), protease (PR) and entry inhibitors, both in vitro [7] and in vivo [8,9]. However, it has been observed that failure of highly active antiretroviral therapy (HAART) including RAL might be related to the emergence of drug-resistant virus variants [10-15], and amino acid changes associated with resistance to integrase inhibitors (INI) have been reported [16-18].

In particular, Y143R/C, N155H and Q148K/R/H have been identified as primary RAL resistance mutations, usually associated with secondary mutations often already present at baseline [10, 12, and 13]. However, the entire panel of mutations associated with RAL resistance has not been fully ascertained. Nor do we fully understand the potential impact of naturally occurring ancillary mutations with respect to: i) promotion of RAL resistance associated mutations, ii) improvement of the activity of mutated IN and iii) HIV-1 replicative capacity. In addition, it is unclear whether drug pressure on the Pol gene by RT and PR inhibitors might influence the emergence of primary or ancillary RAL mutations. Finally, it has been observed that about 20% of new HIV infections are now sustained in Italy by a wide variety of subtype non-B HIV-1 strains and a few HIV-2 strains [19,20]. Thus, it is important to define the variability of the IN gene in treatment naïve and HAART-experienced patients in different HIV-1 subtypes.

The aims of the study were: i) to evaluate IN variability and polymorphism distribution among patients naïve
for RAL treatment; ii) to better understand whether previous HAART treatment not including RAL might be associated with the emergence of mutations conferring resistance to INI; iii) to calculate the genetic barrier of primary and secondary mutations associated with INI resistance in different HIV-1 subtypes.

**Materials and methods**

**Patients**

IN variability was analyzed using stored plasma samples from 95 consecutive patients infected with HIV-1, as well as four HIV-2 positive patients referred to our Institution in the period December 2008 - December 2009. Patients with no available plasma samples or viral load < 1,000 HIV RNA copies/ml plasma were excluded from the analysis. Eligible patients were stratified on the basis of treatment history as follows: i) HAART-naïve patients, ii) RT and PR inhibitor-experienced but RAL-naïve (RTI/PI-experienced).

**Real-time RT-PCR, RT-PCR and sequencing**

HIV-1 plasma RNA levels were determined using the Versant HIV-1 RNA 3.0 Assay (Bayer, NY, USA), while HIV-2 plasma RNA levels were determined according to an in house developed real-time RT PCR [21]. For IN gene sequencing (codons 1-277), a region of the HIV-1 Pol gene was amplified in a nested-RT-PCR using primers Int1F, 5′-CAT GGG TAC CAG CAC ACA CAA AGG-3′ and Int1R, 5′-CCA TGT TCT AAT CCT CAT CCT GTC -3′ for the first PCR round, while primers Int2F 5′-GGA ATT GGA GGA AAT GAA CAA GTA GAT -3′ and Int2R 5′-GCC ACA CAA TCA TCA CCT GCC ATC-3′ were used in the second PCR round [12]. The first nested-RT-PCR reaction was performed in 50 μl using the SuperScript™ III Platinum® One-Step qRT-PCR System (Invitrogen, Carlsbad, CA, USA) with the following thermal profile: 30 min at 50°C and 10 min at 95°C for 1 cycle, 1 min at 95°C, 1 min at 52°C and 1 min and 10 sec at 72°C for 50 cycles followed by 10 min at 72°C. The nested PCR reaction was performed in 100 μl using TaqGold and the relevant buffer (Applied Biosystem, Foster City, CA, USA) with the following thermal profile: 10 min at 95°C for 1 cycle, 1 min at 95°C, 1 min at 50°C, and 1 min and 10 sec at 72°C for 30 cycles, followed by 10 min at 72°C [12]. RT and PR genes were sequenced in parallel [22]. Sequencing of amplicons was performed using an ABI PRISM 3100 Genetic Analyzer® (Applied Biosystem, Foster City, CA, USA) with the ABI PRISM™ Big Dye Terminator Cycle Sequencing Reaction kit.

**Sequence analysis**

IN, RT, and PR sequences were analyzed with the MEGA 4.0 version software [23]. Sequence distances were calculated using the Simmonic sequence editor (version 1.6) program [24], with the Kimura 2-Parameter as a distance estimated method. Divergence was defined as the mean proportion of nucleotide or amino acid differences between all sequence pairs. In each patient, only the predominant virus strain was taken into account to calculate variability. Integrate variability was also calculated in three functional domains: N-terminal domain (NTD), catalytic core domain (CCD), and C-terminal domain (CTD).

**Genotypic resistance and genetic barrier**

Resistance to antiretrovirals was estimated on the basis of the Stanford HIV drug resistance database report (http://hivdb.stanford.edu) and the geno2pheno® report (http://integrase.bioinf.mpi-inf.mpg.de/index.php). Twenty-seven IN positions related to 28 mutations in INI resistant HIV-1 strains were categorized as follows: i) primary mutations (E92Q, F121Y, E138A, G140A/S, Y143R/C/H, S147G, Q148H/R/K, S153Y, N155H, R263K) ii) ancillary mutations (H51Y, T66I, L74A/M/I, Q95K, T97A, E138K, Q146P V151I, E157Q,G163R, I203M, S230R) and iii) mutations with an uncertain role (V72I, T125K, A128Y, K160D, V165I, V201I; http://hivdb.stanford.edu).

The genetic barrier to INI-resistance was calculated in 27 IN amino acid positions with a method previously published by van de Vijver et al. [25]. The smallest number of transitions (scored as 1) or transversions (scored as 2.5) were used to calculate the genetic barrier. The genetic barrier was calculated with the sum of scores obtained for each amino acid position.

**Statistical analysis**

Statistical analyses were performed using GraphPad Prism (version 4.0) software (San Diego, CA, USA). To compare the nucleotide and amino acid divergence between groups of patients the Mann Whitney U-test was utilized, while the chi-square test was used for comparing the calculated genetic barrier for major and minor substitutions between groups of patients.

**Results**

**Study population**

Nucleotide (nt) and amino acid (aa) integrase variability was analyzed in 41 HAART-naïve patients, and 54 RTI/PI-experienced patients. Four HIV-2 strains were also analyzed. Among HIV-1 strains from HAART-naïve patients, 16 were subtype B and 25 were non-B strains (A, no.3; C, no.5; G, no.3; F, no.6; CRF02AG, no.6; CRF01AE, no.1; CRF12BF, no.1). Among HIV-1 strains from RTI/PI-experienced patients, 19 were subtype B and 35 were subtype non-B strains (A, no.1; A/K no.2; C, no.2 D, no.3; G, no.3; D/F, no.1; F, no.11; CRF02AG,
HIV IN, RT, and PR variability

Both IN nucleotide and amino acid variability was higher in HAART-naïve patients with respect to RTI/PI-experienced patients (p < 0.01; Table 1). Conversely, RT and PR nucleotide variability was higher in RTI/PI-experienced patients with respect to HAART-naïve patients (p < 0.01).

Gene variability in the HIV-1 B and non-B subtypes was also analyzed. In subtype B strains, IN amino acid variability was statistically higher in HAART-naïve patients with respect to RTI/PI-experienced patients (6.1 ± 1.5 vs 4.1 ± 1.3; p < 0.001). However, both RT (8.3 ± 2.3 vs 4.5 ± 1.2; p < 0.001) and PR (20.3 ± 7.5 vs 8.9 ± 2.8; p < 0.001) amino acid variability was higher in strains from RTI/PI-experienced patients with respect to HAART-naïve patients. In subtype non-B strains, IN amino acid variability was not statistically different in naïve patients with respect to RTI/PR experienced patients (6.9 ± 1.9 vs 7.1 ± 1.8; p > 0.05), whereas RT (8.0 ± 2.2 vs 7.0 ± 1.9; p < 0.001) and PR (13.3 ± 6.2 vs 10.8 ± 4.1; p < 0.001) amino acid variability was higher in RTI/PI-experienced patients with respect to HAART-naïve patients.

In Table 1, the number of conserved and variable amino acid residues of the IN gene in each group of virus strains is shown. The number of conserved residues in sequences from HAART-naïve patients and RTI/PI-experienced patients was comparable (Table 1). No differences between singleton sites and parsimony informative sites were observed among the variable residues in all patient groups (Table 1).

When the three functional domains of the IN gene were individually analyzed, the amino acid sequences from HAART-naïve patients were slightly more conserved than sequences from RTI/PI-experienced patients. In the analysis of amino acid variability in the three structural domains for all sequence categories, a higher variability in the NTD with respect to the CCD and the CTD was observed (p < 0.001; data not shown).

Frequency, distribution and genetic barrier of IN resistance mutations

In Table 2, mutations in positions associated with INI susceptibility are shown. The eight primary mutations associated with RAL or elvitegravir (EGV) resistance were not present in any of the strains from INI-naïve patients.

HIV-2 variability and mutation distribution

HIV-2 strains showed higher conservation with respect to HIV-1 subtype B and non-B strains (p < 0.001). In detail, in HIV-2 strains, 241/273 (88.3%) conserved sites and 32/273 (11.7%) variable sites were observed (Table 1). The analysis of four HIV-2 integrase genes showed that the mean nucleotide and amino acid variability was 13.0% ± 1.1 and 7.9% ± 1.4, respectively. Three out of four HIV-2 strains analyzed (all from RTI/PI-experienced patients but INI naïve) showed the presence of an E to T polymorphism, in a position where a primary mutation (E138A normally associated with RAL-resistant HIV-1 strains) was detected. Three secondary mutations I72V, E125D and S163D (Table 2) were also detected.

Discussion

The introduction of the new INI antiviral-drug class [6,26] is an important step forward in the treatment of HIV-1 infection [8]. Despite the success of HAART in managing HIV-1 infection, the development and worldwide spread of HIV-1 drug resistant strains remain serious issues. In this study, we analyzed the IN gene variability in parallel with RT and PR variability with the aim of evaluating whether HIV genetic background influences the appearance of IN mutations. The distribution of specific amino acids implicated in INI susceptibility was also compared in different HIV-1 subtypes.
Table 1 Conserved and variable amino acid distribution in Integrase sequences and amino acid divergence among RT, PR and IN sequences in HIV-1

| Category (strain no.) | Mean nucleotide divergence ± SD | Mean amino acid divergence ± SD | IN conserved amino acid (%) | IN variable amino acid (%) | IN conserved amino acid in three functional domains (%) |
|-----------------------|---------------------------------|---------------------------------|----------------------------|---------------------------|-------------------------------------------------------|
|                       | RT gene PR gene IN gene        | RT gene PR gene IN gene         | Singleton\(^a\) Parsimony\(^b\) NTD (1-50) CCD (51-212) CTD (213-277) |                       |                                                      |
| HAART-naive (41)      | 9.2 ± 2.3 10.5 ± 2.9 8.8 ± 2.5 | 6.3 ± 1.8 11.3 ± 3.5 7.0 ± 1.8 | 180 (65.9) 36 (13.2) 57 (20.9) | 26 (52.0) 114 (70.4) 40 (62.5) |
| RT/PI-experienced (54) | 9.8 ± 2.5 12.2 ± 3.6 8.3 ± 2.5 | 9.8 ± 2.6 18.8 ± 7.8 6.5 ± 1.9 | 170 (62.3) 39 (14.3) 64 (23.4) | 26 (52.0) 103 (63.6) 41 (64.1) |
| HAART-naive vs RT/PI-experienced | p < 0.001 p < 0.001 p < 0.001 p < 0.001 p < 0.001 | ns ns ns ns ns | ns ns ns ns ns |
| HIV-2 (4)             | ND ND 13.0 ± 1.1 ND ND 7.9 ± 1.4 | 241 (88.3) 9 (3.3) 23 (8.4) 39 (78.0) 149 (91.9) 53 (82.8) |
| HIV-2 vs HIV-1        | p < 0.001 p < 0.001            | p < 0.001 p < 0.001 p < 0.001 p < 0.001 p < 0.001 |

SD: standard deviation; ND: not done; ns: not significant; NTD: N-terminal domain; CCD: catalytic core domain; CTD: C-terminal domain; RT: reverse transcriptase; PR: protease; IN: integrase.

\(^a\)A singleton site contains at least two types of amino acid with, at most, one occurring multiple times.

\(^b\)Parsimony-informative if it contains at least two types of amino acid, and at least two of these occur with a minimum frequency of two.

\(^{**}\)Significant p-values are reported.
and in patients naïve for treatment or exposed to RTI and PI. The simultaneous evaluation of RT, PR and IN identity showed that the IN gene had lower amino acid variability. This would confirm the high level of integrase sequence conservation reported by Rhee et al. [27]. Moreover, the analysis of three functional integrase domains showed only a small difference in the catalytic core domain.

The evaluation of IN variability in different patient categories showed no differences in subtype non-B and a higher divergence in subtype B strains from HAART-naïve compared with RTI/PI-experienced patients. These results are in contrast with the previously reported greater amino acid IN divergence in RT/PI-experienced patients [28]. On the other hand, we observed a greater RT and PR amino acid divergence in both B and non-B strains from RTI/PI-experienced patients with respect to HAART-naïve patients. These findings are consistent with the hypothesis that multiple rounds of positive selection by subsequent HAART regimens including different RTI and PI, may lead to the emergence of a wider number of RT and PR variants in contrast with little or no change in the IN gene.

In keeping with previous studies [13,29-32] no primary IN mutations associated with INI susceptibility were present in strains from INI-naïve patients.

Table 2 HIV-1 and HIV-2 amino acid polymorphisms at positions associated with INI (RAL and EGV) resistance

| Mutation categories | Known amino acid substitution | Rate of INI resistance mutations in INI-naïve patients (%) | HIV-1 amino acid substitution | HIV-2 clade A ROD subtype consensus | HIV-2 substitution (4 strains) |
|---------------------|-------------------------------|----------------------------------------------------------|--------------------------------|-----------------------------------|-------------------------------|
| Primary mutations   |                               |                                                          |                                |                                   |                               |
| F121Y               |                               |                                                          |                                |                                   |                               |
| E138A               |                               |                                                          |                                |                                   |                               |
| G140A/S             |                               |                                                          |                                |                                   |                               |
| Y143R7C/H           |                               |                                                          |                                |                                   |                               |
| S147G               |                               |                                                          |                                |                                   |                               |
| Q148H/R/K           |                               |                                                          |                                |                                   |                               |
| S153Y               |                               |                                                          |                                |                                   |                               |
| N155H               |                               |                                                          |                                |                                   |                               |
| R263K               |                               |                                                          |                                |                                   |                               |
| Secondary mutations |                               |                                                          |                                |                                   |                               |
| H51T                |                               |                                                          |                                |                                   |                               |
| T66I                |                               |                                                          |                                |                                   |                               |
| L74F/M/I            | 7 (7.4)                       |                                                          | I                              |                                   |                               |
| Q95K                |                               |                                                          |                                |                                   |                               |
| T97A                | 2 (2.1)                       |                                                          | S                              |                                   |                               |
| E138K               |                               |                                                          |                                |                                   |                               |
| Q146P               |                               |                                                          |                                |                                   |                               |
| V151M               |                               |                                                          |                                |                                   |                               |
| E157Q               | 3 (3.2)                       |                                                          | Q                              |                                   |                               |
| G163R               |                               |                                                          |                                |                                   |                               |
| I203D               | 3 (3.2)                       |                                                          | M                              |                                   |                               |
| S230R/M             |                               |                                                          |                                |                                   |                               |
| Polymorphic and non-polymeromorphic mutations | | | | | |
| V72I                | 50 (52.6)                     |                                                          | L                              |                                   |                               |
| T125K               |                               |                                                          | A                              |                                   |                               |
| A128T               |                               |                                                          | A                              |                                   |                               |
| K160D               |                               |                                                          | Q                              |                                   |                               |
| V165I               | 18 (18.9)                     |                                                          | I                              |                                   |                               |
| V201I               | 68 (71.6)                     |                                                          | I                              |                                   |                               |

RTI: reverse transcriptase inhibitor; PI: protease inhibitor; INI: integrase inhibitor. New polymorphisms are reported in boldface.
| IN codon position | Substitution | HAART-naive (n = 41) | RTI/PI-experienced (n = 54) | HAART-naive (n = 41) | RTI/PI-experienced (n = 54) |
|-------------------|--------------|----------------------|-----------------------------|----------------------|-----------------------------|
|                   |              | Codon % distribution | Mutational resistance codon | Lower score | IN codon position | Substitution | Codon % distribution | Mutational resistance codon | Lower scores |
| 51                | H51Y         | CAT 98 100           | TAT 1 143 Y143C TAC 90 90  | TGC 1     |               |
|                   |              | CAC 2 0              | TAC 1 10 TAT 10 10          | GTC 1     |               |
| 66                | T66I         | ACA 98 94            | ATA 1 90 90 Y13R TAC 90 90 | CGC 2     |               |
|                   |              | ACC 0 6              | ATC 1 10 10 TAT 10 10      | CGT 2     |               |
| 72                | V72I         | GTC 15 6             | ATC 1 90 90 Q146P CAA 98 98 | CCG 2.5   |               |
|                   |              | GTA 0 6              | ATA 1 90 90 CAA 98 98      | AAA 2.5   |               |
|                   |              | GTG 0 4              | ATA 2 147 S147G AGT 76 70  | GGT 1     |               |
|                   |              | ATT 41 45            | 0 80 96 CAT/C 2.5          |               |               |
|                   |              | ATC 12 2             | 0 148 Q148H CAA 80 96      |               |               |
|                   |              | ATA 0 4              | 0 80 96 CAT/C 2.5          |               |               |
| 74                | L74I         | CTG 72 78            | ATA 3.5 151 V151I GTA 71 72 |               |               |
|                   |              | CTA 10 15            | ATA 2.5 151 V151I GTA 71 72 |               |               |
|                   |              | TTA 10 2             | ATA 2.5 151 V151I GTA 71 72 |               |               |
|                   |              | TTG 2 0              | ATA 3.5 151 V151I GTA 71 72 |               |               |
|                   |              | ATT 0 0              | 0 153 S153Y TCT 76 87      | TAT 2.5   |               |
|                   |              | ATA 2 7              | 0 153 S153Y TCT 76 87      | TAT 2.5   |               |
|                   |              | ATG 2 0              | ATA 1 15 11 TAC 2.5        |               |               |
|                   |              | GAA 63 61            | CAA 2.5 TCA 7 2 TAT/C 5    |               |               |
|                   |              | GAG 37 39            | CAG 2.5 A153 GCC 2 0 TAC 5 |               |               |
|                   |              | CAA 32 28            | AAA 2.5 155 N135H AAT 90 93 | CAT 2.5   |               |
|                   |              | CAG 68 70            | AAG 2.5 155 N135H AAT 90 93 | CAT 2.5   |               |
|                   |              | CGG 0 2              | AAG 3.5 N135S AAT 90 93    | AGT 1     |               |
|                   |              | ACA 98 96            | GCA 1 AAC 10 7 CAC 2.5     |               |               |
|                   |              | GCA 0 4              | 0 157 E157Q GAA 88 91      | AAA 2.5   |               |
|                   |              | TCA 2 0              | GCA 2.5 GAG 7 7 CAG 2.5    |               |               |
| 92                | E92Q         | GAA 63 61            | CAA 2.5 TCA 7 2 TAT/C 5    |               |               |
|                   |              | GAG 37 39            | CAG 2.5 A153 GCC 2 0 TAC 5 |               |               |
|                   |              | CAA 32 28            | AAA 2.5 155 N135H AAT 90 93 | CAT 2.5   |               |
|                   |              | CAG 68 70            | AAG 2.5 155 N135H AAT 90 93 | CAT 2.5   |               |
|                   |              | CGG 0 2              | AAG 3.5 N135S AAT 90 93    | AGT 1     |               |
|                   |              | ACA 98 96            | GCA 1 AAC 10 7 CAC 2.5     |               |               |
|                   |              | GCA 0 4              | 0 157 E157Q GAA 88 91      | AAA 2.5   |               |
|                   |              | TCA 2 0              | GCA 2.5 GAG 7 7 CAG 2.5    |               |               |
| 121               | F121Y        | TTC 90 98            | TAC 2.5 Q157 CAA 5 2 0     |               |               |
|                   |              | TTT 10 2             | TAT 2.5 160 K160D AAA 85 93 | GAT/C 3.5   |               |
| 125               | T125K        | ACA 27 18            | AAG 2.5 AAG 11 5 GAT/C 3.5 |               |               |
|                   |              | ACT 10 4             | AAA/G 5 R160 AGA 2 0 GAT 4.5 |               |               |
|                   |              | ACG 12 24            | AAG 2.5 Q160 CAA 2 2 GAT/C 5 |               |               |
|                   |              | GCA 44 33            | AAA 3.5 163 G163R GGA 63 57 | AGA 1     |               |
Table 3: Codon distribution and calculated genetic barrier at 27 integrase inhibitor susceptible positions in HIV-1 INI-naïve patients (Continued)

| Position | Codon | Frequency | Genetic Barrier | Calculated Genetic Barrier |
|----------|-------|-----------|----------------|---------------------------|
| V125     | GCG   | 2         | AAG            | 3.5                       | GGG 27 | AGG 1 |
|          | GCT   | 0         | AAA/G          | 6                         | GGT 0  | CGC,AGA/G 3.5 |
|          | GTG   | 0         | AAG            | 3.5                       | E163  | GAA 0  | AGA 2 |
|          | GTA   | 2         | AAA            | 3.5                       | N163  | AAC 0  | CGC,AGA/G 3.5 |
|          | ATG   | 2         | AAG            | 2.5                       | A163  | GCG 0  | CTG,AGG 3.5 |
| P125     | CCG   | 0         | AAG            | 5                         | 165   | GTC 1  | ATT 1 |
|          | GCC   | 37        | ACC            | 1                         | GTC 1  | ATT 1  |
|          | GCT   | 9         | ACT            | 1                         | GTC 1  | ATT 1  |
| 128      | A128T | 54        | ACA            | 1                         | ATA 20 | ATA 2  |
|          | GCA   | 50        | AAA            | 1                         | I165  | ATA 18 | 0   |
|          | GCC   | 37        | AGC            | 1                         | I165  | ATA 70 | 0   |
|          | GCT   | 9         | ACT            | 1                         | I165  | ATA 70 | 0   |
| 138      | E138K | 100       | AAA            | 1                         | I165  | ATA 20 | 0   |
| D138     | GAC   | 0         | AAA/G          | 2                         | V201I | GTA 24 | 1   |
| 140      | GGA   | 46        | AGT/C          | 3.5                       | GTG 2  | ATA 2  |
|          | GCC   | 46        | AGC            | 1                         | I201  | ATA 70 | 0   |
|          | GGT   | 5         | AGT/C          | 1                         | I201  | ATA 70 | 0   |
|          | GGG   | 5         | AGT/C          | 3.5                       | M203  | AGT 5  | 0   |
| G140A    | GGA   | 46        | GCA            | 2.5                       | S230R | AGC 96 | 1   |
|          | GCC   | 46        | GCT            | 2.5                       | N230  | AAC 10 | 0   |
|          | GGT   | 5         | GCT            | 2.5                       | R263K | AGA 85 | 1   |
|          | GGG   | 6         | GCT/G          | 2.5                       | AGG 15 | AAA 1  |

The amino acids differing from wild-type or expected mutant are in boldface.
However, in sequences from INI-naïve patients some secondary mutations, which have been shown to contribute to RAL resistance [28-31], were found. The low prevalence and the equal distribution of these polymorphisms among the different groups of patients are in contrast with the reported appearance of secondary mutations in association with prior antiretroviral exposure [28,31]. Thus, our results are in accordance with findings by Garrido et al. [32] and suggest that the emergence of RAL resistance mutations is weakly influenced by prior exposure to antiretrovirals other than INI.

Finally, new polymorphisms (Q95R, and T97S) in positions related to INI resistance were found. Whether and how these mutations might influence viral fitness or replication remains to be clarified.

The genetic barrier was calculated for 27 amino acid positions related to INI susceptibility. The majority of these positions were highly conserved. Our analysis extends the results reported by Maiga et al [33] (including only B and CRFD02 AG subtypes) to a wider collection of HIV-1 subtypes which reflects the evolving epidemiology of this infection in our region [19]. Analysis of codon usage distribution between sequences from HAART-naïve and RTI/PI-experienced patients revealed a single position (148), with a predominant difference in codon usage. These findings suggest a marginal yet valid influence of prior antiretroviral exposure on the genetic barrier in our study population [33]. A larger dataset would allow better definition of the role played by previous treatment with RTI and PI on INI susceptibility. On the other hand, the great majority of patients on HAART show complete suppression of peripheral viral load, and the enrollment of 95 viremic patients required 1 year to be completed.

Due to the small number of HIV-2 sequences, our analysis did not allow us to draw conclusions on HIV-2 variability. However, of particular interest was the detection of mutations in IN positions associated with RAL resistance. This finding confirms and extends a previous observation by Xu et al. [34]. The identification of INI resistance mutations in INI-naïve patients infected with HIV-2 highlights the urgent need for future studies on HIV-2 and may necessitate avoidance of INI in the treatment of these patients.

In conclusion, primary INI resistance-associated mutations were not present in this population of INI naïve HIV-1 infected individuals. Exposure to antivirals other than INI does not seem to significantly influence the emergence of mutations implicated in INI resistance.

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Authors’ contributions
AP has made great contribution to sequences analysis and manuscript preparation. SP and RG have been involved in sample collection and sequencing. GC has been involved in sample collection. FB has contributed in manuscript preparation and fund raising.

All authors read and approved the final manuscript.

Competing interests
The authors declare that they have no competing interests.

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