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TRANSMITTED DRUG RESISTANCE MUTATIONS AND TRENDS OF HIV-1 SUBTYPES IN TREATMENT-NAÏVE PATIENTS: A SINGLE CENTER EXPERIENCE

Laura Mazzuti[1], Taulant Melengu[1], Francesca Falasca[1], Marianna Calabretto[1], Eleonora Cella[2], Massimo Ciccozzi[2], Ivano Mezzaroma[3], Giancarlo Iaiani[4], Martina Spaziante[5], Gabriella d'Ettorre[5], Caterina Fimiani[4], Vincenzo Vullo[5], Guido Antonelli[1], Ombretta Turriziani[1].

[1]Department of Molecular Medicine, Sapienza University of Rome, Italy

[2]Unit of Medical Statistics and Molecular Epidemiology, University Campus Bio-Medico of Rome, Italy.

[3]Department of Translational and Precision Medicine Department of Public Health and Infectious Diseases, Sapienza University of Rome, Italy

[4] Umberto I University Hospital, Rome, Italy

[5]Department of Public Health and Infectious Diseases, "Sapienza" University of Rome, Italy

Highlights

- Primary drug resistance can reduce the efficacy of antiretroviral therapy
- HIV-1 non-B subtypes are increasing in Italy
- The frequency of transmitted drug resistance mutations remains stable over the years
- Major mutation to Integrase inhibitors in naive patients was found for the first time in Italy

ABSTRACT

Background: Transmitted drug resistances (TDRs) and HIV-1 diversity could affect treatment efficacy and clinical outcomes. Here we describe the circulating viral subtypes and estimate the

prevalence of resistance among drug naïve patients attending Sapienza University Hospital in Rome from 2006-2017.

Methods: Genotypic resistance test (GRT) was performed on 668 ART-naïve patients. GRT were conducted in integrase (n= 52), protease and reverse transcriptase (n=668) sequences.

Results: Twenty-one different subtypes and Circulating Recombinant Forms (CRFs) were identified. Subtype B was the most common (67%), followed by CRF02_AG (8.3%), subtypes C and F (6%). We found a significantly increased overtime in the proportion of non-B strains and in the rates of non-Italian patients (p<0.001). The overall prevalence of TDR was 9.4% (NRTI: 4.2%, NNRTI: 5.8%, PI: 1%) and was higher in B strains. Transmitted INSTI mutations, Q148H and G140S, responsible for high-level of resistance to raltegravir and elvitegravir and intermediate resistance to dolutegravir and bictegravir were found, for the first time, in two individuals. Minor or accessory INSTI mutations were detected in 17.3% of patients. No significant decrease of TDR prevalence was documented overtime.

Conclusion: The significant increase of non-B subtypes suggests that the molecular epidemiology of HIV-1 is changing. The detection of a major INSTI mutation in two naïve patients highlights the importance of performing GRT before commencing treatment. This finding and the lack of a significant reduction of TDR underline the importance of a continuous surveillance of resistance mutations.

Key words: Resistance mutations, transmitted drug resistance, antiretroviral naïve patients, HIV-1 subtypes, integrase inhibitors resistance

Introduction

The use of antiretroviral therapy (ART) for the treatment of HIV-1 infection in developed countries has been accompanied by an increase in transmitted drug resistances (TDRs) in ART-naïve patients [1], limiting the choice of first line antiretroviral drugs [2,3]. The emergence of TDR because of

ART expansion represents a serious public health issue because TDRs may affect treatment efficacy and may negatively affect individual's prognosis. Despite that Italian and European guidelines for the use of antiretroviral agents and the diagnostic-clinical management of HIV-1 infected persons recommend performing Genotypic resistance test (GRT) prior to initiation of ART [4-6], the prevalence of TDR mutations has remained stable, at 8%-10% approximately, in these countries over the years [7].

In addition, since integrase strand transfer inhibitors (INSTIs) are part of recommended first-line regimens for the treatment of HIV-1 infection, INSTI mutation surveillance gains importance in order to optimize the efficacy of the therapy. Primary INSTIs resistance is still rare but reports of TDRs to these drugs are emerging [8-11]. Nonetheless, baseline resistance to this class is still not routinely performed and nowadays only few studies evaluate the prevalence of INSTI mutations in naive patients [12-14]. To date the presence of pretreatment INSTI resistance among ART naïve HIV-1 infected patients in Italy was never reported.

Moreover, several authors reported epidemiological changes, such as an increasing trend of non-B subtypes and circulating recombinant forms (CRFs) in Europe, Australia and North America [15-18]. This increase underlines that geographical patterns in subtype distribution are changing over time, due to migration and the mixing of population [15,16]; this phenomenon is clinically relevant, because these changes can have an impact on pathogenesis, resistance pathways, disease progression, diagnosis and vaccine development [17].

The aim of this study was to examine temporal changes in subtype diversity and to evaluate the prevalence of TDR among ART naïve newly diagnosed HIV-1 infected individuals in 2006-2017.

Methods

Study population

A retrospective study was conducted on 720 GRTs (668 *pol* sequences and 52 *integrase* sequences) from 668 ART-naïve patients attending Umberto I Sapienza University Hospital from 2006 to 2017. For each patient the GRT performed for routine clinical purposes at diagnosis or prior to the start of therapy was considered. Baseline demographic data from studied individuals were summarized in table 1.

Informed consent was obtained according to the standards of the local ethics committee. All information, including virological, clinical and demographic data, was recorded in an anonymous database.

Genotyping and drug resistance mutations analysis

GRTs from plasma samples (*pol* and *integrase* genes) were performed until 2015 using TruGene® HIV-1 Genotyping Kit (Siemens Healthcare Diagnostics Inc., Deerfield, IL), and TruGene Core Reagent as previously described [19-20]; then ViroSeq™ HIV-1 Genotyping System (Celera Diagnostics, Alemada, CA) and ViroSeq™ HIV-1 Integrase RUO Genotyping Kit (Celera, US) were used according to the manufacturer's instructions.

The HIV-1 subtypes and circulating recombinant forms (CRFs) were determined according to the Stanford University HIV Drug Resistance Database HIVdb Program (version 8.5, https://hivdb.stanford.edu/hivdb).

TDR mutations were defined as the presence in the *pol* region of at least one major mutation included in the International AIDS Society (IAS) list [21] and/or the Stanford University HIV Drug Resistance Database HIVdb Program (version 8.5, https://hivdb.stanford.edu/hivdb). As far as INSTI mutations are concerned, all mutations reported in IAS and Stanford list were considered.

HIV-1 strains were defined as resistant if carrying at least one TDR mutation. The overall prevalence was defined as the percentage of patients infected with a virus carrying any drug resistance mutation (DRM). The prevalence of TDR for the different drug classes [nucleoside RT

inhibitors (NRTI), non-nucleoside RT Inhibitors (NNRTI), protease inhibitors (PI) and INSTI), was defined as the percentage of patients infected with a virus carrying any DRM associated with each drug class.

Phylogenetic analysis

Phylogenetic relationships were analyzed by constructing a Maximum Likelihood phylogenetic tree by IQTREE [22]. The best substitution model (GTR+I+G) was selected by analysis of sequences with the Models tool in MEGA. Tree reliability was assessed by setting bootstrap replicates to 1000. Bootstrap values > 70 were considered significant. The tree was rooted with midpoint rooting and edited using FigTree v1.4.0.

To investigate the demographic history of the naïve couple (subtype B), Bayesian calculations were performed by calibrating a molecular clock using known sequences sampling times with the Bayesian Markov Chain Monte Carlo (MCMC) method implemented in BEAST v. 1.8.2 (http://beast.bio.ed.ac.uk) [23,24]. Independent MCMC runs were conducted for at least 100x 106 generations and sampled every 10000 steps for each molecular clock model. Convergence of the MCMC was assessed by calculating the ESS for each parameter. Only parameter estimates with ESS's of >250 were accepted. The maximum clade credibility (MCC) tree was obtained from the trees posterior distributions, after a 10% burn-in, with the Tree-Annotator software v 1.8.2, included in the BEAST package [23,24].

Statistical analysis

The distribution of studied subjects regarding categorical parameters was compared using χ^2 test. To evaluate potential differences in trends over time χ^2, test for trends was used. A P-value of <0.05 was considered statistically significant. All analyses were carried out using Statistical Package for the Social Science (SPSS, 18.0) for Windows (Chicago, IL, USA) and EPI INFO, version 7.2.2.6.

Results

Patients characteristics

Between 2006 and 2017, 668 HIV-1–infected ART-naive individuals underwent genotypic resistance testing. The demographic characteristics of these subjects are shown in Table 1. The patients were primarily male (75.7%), of Italian origin (69.6%), with a median age of 38 years (IQR 31- 48); the most common risk factors were homosexual (44.2%) and heterosexual contact (43.4%). At the time of GRT, the median CD4 count was 340 cells/mm3 (IQR 148-570) and the median plasma HIV-1 RNA level was 4.71 $\log_{10}$ copies/mL (IQR 4.1-5.3).

Twenty-one different subtypes and Circulating Recombinant Forms (CRFs) were identified. Subtypes B viruses was the most commonly detected (66.9%; 448/668), followed by CRF02_AG (8.3%; 56/668), subtypes C and F (6%; 40/668).

Over the 12 year-study period, there was a significant increase in non -B subtype infection (13.6% in 2006 vs 51% in 2017; p <0.001) (figure 1a) and an increase in the rate of foreign-born population (p=0.043). Higher percentages of non-B infections occurred among persons born abroad (55.72%) compared with those born in Italy (22.67%) (p<0.001). Nevertheless, an increase in the rate of Italian patients infected with a non-B virus (9.0% in 2006 vs 27.0% in 2017; p<0.001) was also found (Figure 1b).

Prevalence of *pol* drug resistant mutations

GRTs before initiation of ART were available in 668 patients. From 2006 to 2017, the overall prevalence of patients with at least one TDR *pol* mutation was 9.4% (63/668). Forty-two out of six hundred-sixty-eight patients (6.3%) had 1 drug class mutation, 11 had more than one mutation associated to resistance to one class drug and 10 individuals harbored multiclass resistant virus (1.4%). Specifically, dual-class TDR was 0.9% (6/668) for NRTI+NNRTI and 0.45% (3/668) for NRTI+PI. Only one patient infected, with subtype F1, had a virus with triple class drug resistance (table 2). Overall, resistance to NNRTI was the most common (5.8%; 39/668 patients), followed by

NRTI (4.2%; 28/668) and PI (1.05%; 7/668). As expected, prevalence of TDR to NNRTI and NRTI were significantly higher than PI ($p<0.001$).

The most frequent NNRTI-associated mutation was K103N, detected in 13 patients (1.9%), followed by E138G/K/Q (7/668, 1%), V108I (6/668, 0.9%) and Y181C (5/668, 0.7%). For NRTI resistance, thymidine analogue mutations (TAMs) occurred more frequently with T215D/S mutations detected in 1.5% of patients (10/668), followed by D67N and M41L (4/668, 0.6%). PI-related mutations were rare, M46I was found in 3 individuals as well as I54M/L/V (0.4%) (Figure 2).

Despite the presence of some fluctuations over the years, the overall prevalence of TDR remains at around 9.4% and did not significantly change overtime (Figure 3).

The detection rate of TDR in B subtype infected patients was higher than in those infected with non-B subtypes (11.6% versus 6%, $p=0.03$).

Prevalence of integrase strand inhibitor mutations

HIV-1 integrase mutation data were available for 52 ART-naïve patients collected between 2009 and 2017. Most of them were infected with subtype B (71.1%; 37/52).

Minor or accessory mutations were detected in 9 patients (17.3%). Among the minor mutations, T97A and E157Q were detected in 5.7% of patients, G140S accounted for 3.8% of INSTI mutations and E138K occurred in one individual (1.9%).

Two patients had the major Q148H mutation and the minor G140S. Interestingly, these two individuals were in an acute phase of infection when the genotypic test was performed; they were a couple and both active drug users, and share also the RT mutations (E138G, T215S, H221Y, M230L).

Phylogenetic analysis

From Maximum Likelihood tree performed on the HIV-1 naïve subtype B sequences, five statistically supported clades have been highlighted. The clusters mainly included sequences from different years. Mutated viruses were dispersed among clade, except some viruses probably originated from the same strain.

Phylogenetic trees demonstrated that the viruses from the HIV-1 naïve couple were almost identical. The BSP (Bayesian Skyline Plot) growth demographic model with a relaxed molecular clock was selected as the most appropriate to describe the evolutionary history of this virus. The evolutionary rate used for the Bayesian calculation was 0.0021 substitutions site per year. The root of the time of the most common recent ancestor (tMRCA) corresponding to January 2017 (May 2016 - February 2017) indicating that the probable origin of that strain dated back to early 2017.

The Maximum Likelihood phylogenetic tree of HIV-1 non-B subtype showed 39 statistically supported clusters (bootstrap>70%). Specifically, subtype CRF01_AE and F showed 7 clusters, subtype-G and subtype-C showed, respectively, 6 and 4 clusters; 9 clusters were identified for CRF02_AG, and 3 clusters were detected for subtype A and CRF12_BF.

Discussion

This study focused on the occurrence of TDR in 668 HIV-1 drug naïve infected individuals attending Umberto I Sapienza University Hospital in the period 2006-2017.

Our treatment-naïve population, as reported in other Italian cohorts [25-28], showed a significantly increased proportion of patients carrying non-B subtype and an increased rate of the foreign-born population in the last years. As expected, we found a higher prevalence of non-B infections among non-Italian patients. This is in line with the data reported in several European countries during the last decade and can be explained by the waves of migrant from low-middle income areas [28-31]. However, we also found a significant increase of non-B infections in Italian patients in more recent years. This finding, in agreement with previous observation [27,28], clearly indicates that, in

conjunction with epidemiological changes, non-B strains have become endemic in the Italian population.

In our center, the estimated prevalence of TDR was 9.43% as the global incidence in Italy [14,32] and was significantly higher in subtype B than in non-B. This data may be due to a different selective pressure exerted by drugs on B and non-B subtype overtime probably because of limited ART coverage in the country of origin of non-B infected strains. Currently, NNRTI remains the drug class with the highest prevalence of TDR mutations [33,34]. The frequency calculation of single resistance mutations revealed that K103N, a non-polymorphic mutation that causes high-level resistance to NVP and EFV, was the most frequently observed. Probably this occurs because this mutation can be quickly transmitted due its limited effect on the viral fitness [35]. The persistence of DRM in the naïve population could also be associated with a high use of the drug responsible for its selection. It is known that K103N is selected in patients receiving first-generation NNRTI, such as NVP and EFV, that are drugs with a low genetic barrier. NVP was widely used in the past years especially before the introduction of the second-generation drugs, rilpivirine and etravirine, while EFV is still recommended as an alternative in Italian and European guideline [4-6]. The detection of K103N, despite the introduction of new drugs over the years, suggests that its persistence is not linked to the continuous use of its selective agents. Similarly, among NRTI DRMs, T215 revertant mutations, such as T215D/S, and M41L were most frequently detected. These mutations belong to the TAMs pattern and are selected by thymidine analogs a class of drugs no longer used in current therapeutic regimens. Therefore, despite a progressive reduction of drugs selecting TAMs and K103N, these mutations remain the most frequent in ART-naïve patients [16,27,28], suggesting that these viral variants are well adapted to host and derive from individuals who had been infected for a long time and treated in the past with suboptimal therapy.

In the current study the prevalence of INSTI TDR was also analyzed in a small number of samples. ART containing INSTI has become the preferred first-line regimen, as currently recommended by

ART guidelines in high-income countries [4-6]. However, there is no consensus opinion regarding baseline testing for INSTI resistance, basically because INSTI resistance remains rare worldwide.

This study provides important information about TDRs to INSTI in Italy, because to the best of our knowledge, it documents for the first time the presence of major associated INSTI DRMs in Italian ART naive patients. We identified a virus with Q148H and G140S mutations, responsible for a high-level of resistance to raltegravir and elvitegravir and intermediate resistance to dolutegravir and bictegravir. Interestingly, naïve patients who harbored this virus, were a couple, both intravenous drug users and in an acute phase of infection. Phylogenetic analysis revealed that these patients harbored the same viruses and that the infection dates back to early 2017. In literature, few cases of transmitted INSTI resistance have been reported [8-10, 36] and recently, a case of transmitted INSTI resistance affecting second-generation INSTI, dolutegravir and bictegravir [11], has been documented.

Moreover, in our small cohort, minor INSTI mutations were observed in 17.3% of analyzed sequences. The prevalence of these minor and accessory substitutions was lower than that detected in another cohort [37], but higher than that reported elsewhere [13]. To date, the clinical impact of these mutations in naive patients starting an INSTI containing regimen is unknown and prospective studies are needed to elucidate their role in affecting genetic barrier.

In conclusion, although our study was limited by the single-center setting, our data revealed that, despite subtype B continues to predominate in Italy, the percentage of non-B infections has grown in recent years, also among Italian patients. These data emphasize that the molecular epidemiology of HIV-1 in Western Europe, including Italy, is changing and underline the importance of monitoring the dynamics of HIV-1 transmission. The broad genetic diversity of HIV-1 can have important implications for public health since subtypes and CRFs can show different properties that can affect their fitness, transmissibility, and response to therapy [38].

Our study also shows that the prevalence of TDR appears to be stable despite the availability of newer antiretroviral drugs and pre-therapy GRT recommended, that should help to prevent virological failure and accumulation of further DRM.

Finally, our study, even though performed on a number of samples too low for a prevalence study, highlights the importance of implementing data about resistance to INSTI in newly diagnosed population and understanding the impact of the minor INSTI mutations in this population. Despite the significant role of INSTIs as first-line ARV agents for the treatment of HIV-1 infection, the documented cases of TDRs to INSTI should serve as a reminder that the appearance of resistance is always lurking. INSTI have been increasingly used for both first-line and salvage ART, therefore it is reasonable to assume that the prevalence of INSTI mutations might rise over time. Our study reinforces the current recommendations to perform GRT for integrase inhibitors in ART naïve patients, especially in those presenting TDR to other classes of drugs, in order to guide treatment decisions.

Declarations

Funding: No funding

Competing Interests: No conflict of interest

Ethical Approval: Informed consent was obtained according to the standards of the local ethics committee

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Legend to figures

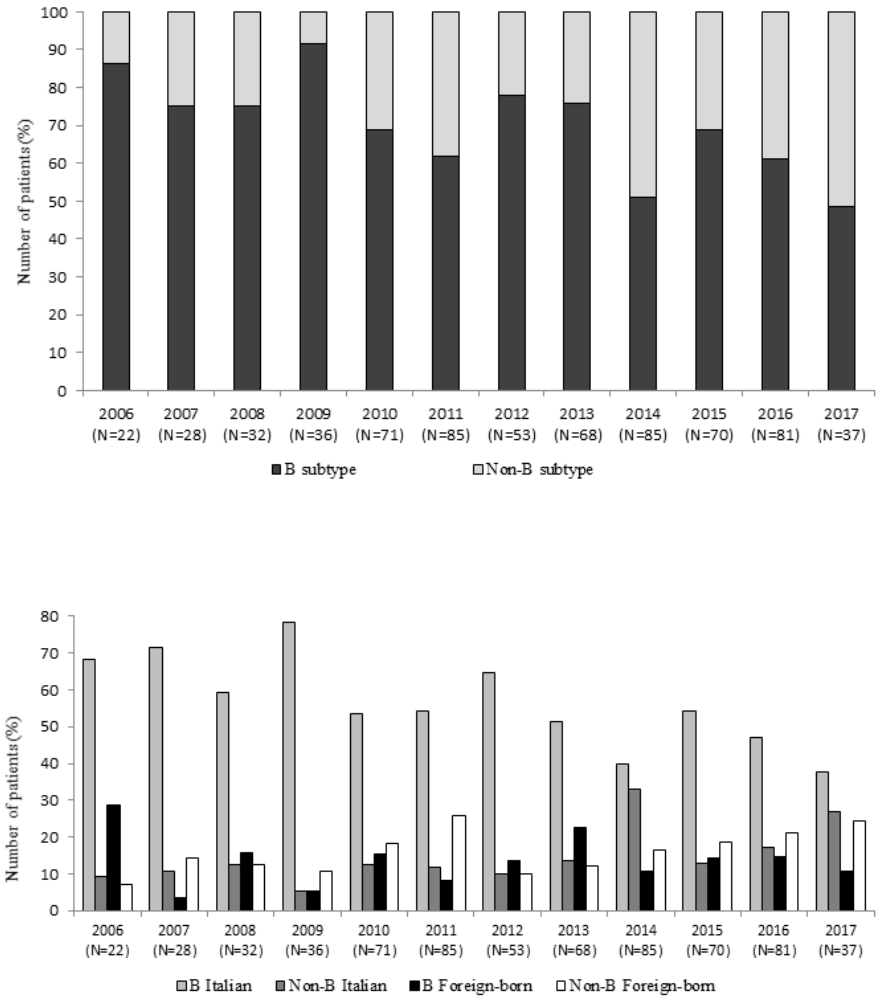

Figure 1. Prevalence of HIV B and non-B subtypes over time in the overall population (A) and in Italian or foreign-born patients (B).

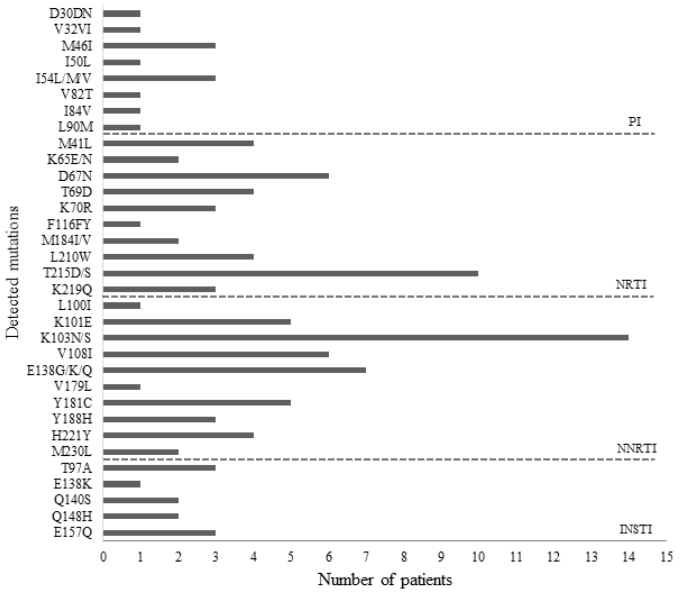

Figure 2. Transmitted drug resistance mutations detected in naïve patients

PI: protease inhibitor; NRTI: nucleoside reverse transcriptase inhibitors; NNRTI: non-nucleoside reverse transcriptase inhibitors: PI: protease inhibitors; INSTI: integrase strand transfer inhibitors

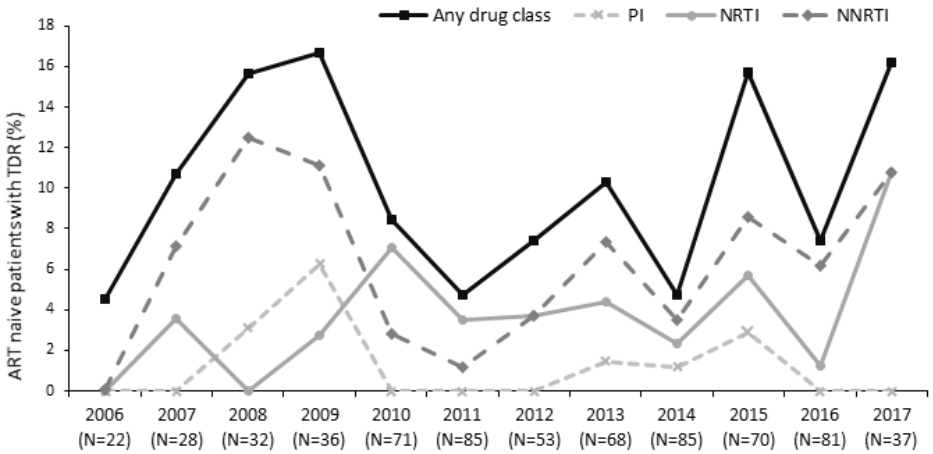

Figure 3. Temporal trends in the yearly proportion of transmitted drug resistance mutations among overall naive population.

PI: protease inhibitor; NRTI: nucleoside reverse transcriptase inhibitors; NNRTI: non-nucleoside reverse transcriptase inhibitors: PI: protease inhibitors

Table 1. Characteristics of the HIV-1 infected patients included in the study

All subjects, n (%)	668
Male, n (%)	506 (75.7)
Age at diagnosis, median (IQR)	38 (31-48)
CD4 count (cell/µL), median (IQR)	340 (148-570)
HIV RNA load (log copies/mL), median (IQR)	4.71 (4.1-5.3)
Country of origin, n (%)	
Italy, n (%)	465 (69.6)
Foreign country, n (%)	203 (30.3)
Route of transmission	
MSM/bisexual, n (%)	295 (44.1)
Heterosexual, n (%)	290 (43.4)
Injecting drug user, n (%)	59 (8.8)
Other	24 (3.6)
Viral subtype, n (%)	
A	19 (2.8)
B	448 (67)
C	40 (6.0)
F	40 (6.0)
CRF02_AG	56 (8.4)
Others	65 (9.7)