Journal of Global Antimicrobial Resistance 41 (2025) 68–76

Contents lists available at ScienceDirect

Journal of Global Antimicrobial Resistance

journal homepage: www.elsevier.com/locate/jgar

Next-generation sequencing and drug resistance mutations of HIV-1 subtypes in people living with HIV in Sicily, Italy, 2021–2023

Luca Pipitò[a,b,c], Marcello Trizzino[b,c], Chiara Mascarella[a,d], Sara Cannella[a,d], Roberta Gaudiano[a,b,c], Irene Ganci[a,b,c], Gaetano D'Alessandro[a,d], Benedetta Romanin[b,c], Maria Mercedes Santoro[e], Giovanni M. Giammanco[a,d], Antonio Cascio[a,b,c,*], Celestino Bonura[a,d]

[a] Department of Health Promotion, Mother and Child Care, Internal Medicine, and Medical Specialties "G D'Alessandro," University of Palermo, Palermo, Italy
[b] Infectious and Tropical Diseases Unit and Sicilian Regional Reference Center for the fight against AIDS, AOU Policlinico "P. Giaccone," Palermo, Italy
[c] Palermo Fast-Track City, Casa dei Diritti, Palermo, Italy
[d] Microbiology and Virology Unit, AOU Policlinico "P. Giaccone", Palermo, Italy
[e] Department of Experimental Medicine, University of Rome Tor Vergata, Rome, Italy

ARTICLE INFO

Article history:
Received 29 July 2024
Revised 11 December 2024
Accepted 15 December 2024
Available online 27 December 2024

Keywords:
HIV drug resistance testing
Genotypic resistance testing
HIV subtypes
Next-generation sequencing
Virological failure
Drug resistance mutations

ABSTRACT

Objectives: HIV-1 infection continues to be a significant public health concern, notwithstanding the expanded utilization of antiretroviral treatment (ART), due to the emergence of drug resistance. The prevalence of transmitted drug resistance remains uncertain, particularly concerning integrase inhibitors. This study aimed to assess the extent of HIV resistance in both ART-naïve and experienced individuals living with HIV (PLHIV) at the University Hospital in Palermo, Italy.

Methods: Genotyping and mutation analysis were performed on ART naïve and experienced PLHIV admitted from June 2021 to October 2023 by the NGS method. Mutations were detected by testing different NGS frequency cut-offs: ≥5 %, ≥10 %, and ≥20 %. Demographic, clinical, virological, and immunological data were retrospectively collected.

Results: Of the PLHIV, 85 (70 %) were ART-naïve, while 36 (30 %) were ART-experienced with virological failure. The main HIV-1 subtype was B (54 %), which was significantly associated with Italy-born ($P < 0.001$) and experienced PLHIV ($P = 0.024$). In the remaining cases, A1 (6 %), C (3 %), F1 (7 %), G (2 %), and Circulating Recombinant Forms (28 %) were reported. At least one mutation for a drug class was detected in 39.7 %, 45.4 %, and 53.7 % of cases at HIV-1 NGS thresholds of 20 %, 10 %, and 5 %, respectively. Drug resistance was found in 18.2 %, 25.6 %, and 33.0 %, by NGS cut-off of 20 %, 10 %, and 5 % respectively. The lowering of NGS cut-offs mainly increased the rates of integrase strand transfer inhibitor resistance. For overall resistance, no difference was observed between B and non-B subtypes for any NGS cut-offs.

1. Introduction

Human immunodeficiency virus type 1 (HIV-1) infection continues to be a major public health concern. The World Health Organization estimated 39 million people living with HIV (PLHIV) and 630.000 associated deaths worldwide in 2022 [1]. HIV-1 is characterized by high genetic diversity due to the high mutation rate induced by the HIV reverse transcriptase enzyme and its high rates of viral replication [2,3]. The increased accessibility of antiretroviral therapy (ART) improved the quality of life and life expectancy of PLHIV. However, widespread use of antiretrovirals was associated with the emergency of drug resistance. Therefore, the HIV genotypic resistance test (GRT) still today remains an essential tool for the management of PLHIV [4].

For HIV-1 drug resistance mutation detection, next-generation sequencing (NGS) is gradually replacing Sanger sequencing as the primary method for HIV GRT. Unlike the Sanger method, NGS

* Corresponding author. Mailing address: Infectious and Tropical Disease Unit, AOU Policlinico "P. Giaccone", Via del Vespro 129, 90127 Palermo Italy.
E-mail addresses: antonio.cascio03@unipa.it, antoniocascio1@gmail.com (A. Cascio).

https://doi.org/10.1016/j.jgar.2024.12.015

enables the simultaneous sequencing of millions of DNA fragments, providing a comprehensive genomic view and detecting less-represented mutations and variants [5,6]. In fact, the Sanger method allows the detection of only major variant mutations, having an intra-host frequency above a threshold of approximately 20 %, while NGS exhibits notably higher sensitivities for identifying resistance variants with a lower frequency [6,7]. Globally, there are limited data on the prevalence of drug mutations and resistances detected using NGS, and no data are reported for Sicily, Italy.

This study aimed to describe the HIV-1 subtype diversity and the prevalence of mutations detected by NGS at different cut-offs in a cohort of ART-naïve and experienced PLHIV followed-up at Paolo Giaccone University Hospital in Palermo (Sicily, Italy), whose GRT was performed between June 2021 and October 2023.

2. Methods

2.1. Study population

A cross-sectional study was conducted, including all PLHIV for whom a GRT was performed at the University Hospital Paolo Giaccone in Palermo (Italy) between June 2021 and October 2023. The Palermo Polyclinic is home to the Regional Reference Center for the fight against AIDS. This is the center where the largest number of PLHIV are followed in Sicily. PLHIV were subdivided into ART-naïve and treatment-experienced, who had suffered a virological failure. Virological failure was defined as an HIV-1 RNA level above 200 copies/mL in PLHIV that initially achieved viral suppression (HIV-1 RNA levels below 50 copies/mL). Data about age, country of origin, ART history, HIV viral load, and CD4 T cell count at the time of GRT were collected for each group retrospectively and recorded in an anonymous database.

2.2. Genotyping, subtyping, and drug susceptibility

GRT was performed for protease, reverse transcriptase, and integrase genes for routine clinical purposes using viral RNA extracted from plasma samples. The commercial kit AD4SEQ HIV-1 Solution v2 (Arrow Diagnostics S.r.l., Genova, Italy) was used with the MiSeq Illumina platform for NGS analysis. After sequencing, the raw FastQ files were analyzed by using the dedicated standalone software SmartVir (SmartSeq s.r.l., Alessandria, Italy) provided with the commercial kit which, by querying the free web tool HIVdb ver 9.5.1 [8], returns a report with all the mutations identified and any drug resistance-associated. Subtype was assessed by using the same software SmartVir, which generates the result always by querying the HIVdb ver 9.5.1 algorithm. Initially, the prevalence of overall mutations (major, minor, and accessory) was reported, including mutations not associated with drug resistance. Subsequently, drug resistance mutations and high-level resistance to the specific drugs were evaluated according to the HIVdb program integrated into the HIV-1 drug resistance database v.9.5.1. Analyses were performed by testing different NGS frequency cut-offs: ≥5 %, ≥10 %, and ≥20 %. ART-naïve and experienced PLHIV and B and non-B HIV-1 subtypes were compared for the prevalence of mutations and drug resistance.

2.3. Statistical analysis

Analyses were performed using the SPSS v.26.0 software package for Windows (IBM SPSS Statistics, ver. 26.0). Continuous variables were summarized as median and interquartile range and categorical variables as absolute and relative frequencies. Differences in the median were evaluated using the Mann-Whitney U test; the $\chi 2$ test or Fisher's exact test was applied to categorical variables as appropriate.

3. Results

3.1. PLHIV characteristics

Demographic and virological-immunological characteristics of the PLHIV included in the study are shown in Table 1. Of the participants, 85 (70 %) were ART-naïve, while 36 (30 %) were ART-experienced with virological failure. Five ART-naïve individuals repeated the GRT due to virological failure and were therefore recategorized into the ART-experienced group. The majority of PLHIV were of Italian origin (78 %). The remaining countries of origin included Africa (17 %), South America (2 %), Eastern Europe (2 %), and Southeast Asia (1 %). Non-Italian PLHIV were more commonly female. Treatment-naïve PLHIV were younger than ART-experienced PLHIV and had higher HIV viral loads.

Diagnosis of acquired immunodeficiency syndrome (AIDS) was reported in 36 (42 %) ART-naïve subjects. The most common AIDS-defining conditions were wasting syndrome (25 %) and pneumocystis pneumonia (25 %), followed by candida esophagitis (19 %), tuberculosis (14 %), Kaposi sarcoma (11 %), *Toxoplasma gondii* encephalitis (8 %), progressive multifocal leukoencephalopathy (8 %), and cryptococcosis (5 %). Other AIDS-defining conditions were cytomegalovirus-related gastritis, human papillomavirus-related anal carcinomas, non-Hodgkin lymphoma, progressive disseminated histoplasmosis, and HIV-related encephalopathy, in one case each.

In ART-experienced individuals, therapies at failure before GRT were based on an integrase strand transfer inhibitor (INSTI) regimen in 21 cases, followed by a protease inhibitor (PI) associated with INSTI in 6 cases and PI alone in 6 cases. NNRTI, INSTI associated with NNRTI, PI associated with NNRTI, and INSTI combined with PI and NNRTI were reported as failures in one case each. In all cases, virological failure was related to low compliance with ART. For treatment-experienced PLHIV, the start date of ART was available for 94.4 % of the cases, with the median duration from the start of ART to the day of GRT at failure being 145 months (IQR 82–281). No significant associations were found between the duration of HIV infection and resistance to specific drug classes. The history of previous ART regimens was available for 34 PLHIV, with 86.1 % having included INSTIs, 61.1 % PIs, and 13.9 % NNRTIs. The use of at least two drug classes among INSTI, PI, and NNRTI was reported in 55.5 %. In 4 cases, the ART history included the use of INSTI, PI, and NNRTI.

3.2. HIV-1 subtypes

HIV-1 subtype was determined for 120 individuals. The main HIV-1 subtype was B (54 %), which was significantly associated with Italian-born and experienced PLHIV (Table 2). In the remaining 46 % of cases A1, C, F1, G, and several Circulating Recombinant Forms (CRF) were reported. CRFs were significantly associated with non-Italian PLHIV. The most common recombinant was CRF02_AG (27 cases), followed by CRF06_cpx and CRF09_cpx (two cases each), and CRF13_cpx, CRF12_BF, and CRF20_BG (one case each).

3.3. Prevalence of mutations and drug resistance at 20 %, 10 % and 5 % NGS frequency cut-offs

Among the 121 GRTs, at least one mutation for a drug class was detected in 39.7 %, 45.4 %, and 53.7 % of cases at HIV-1 NGS thresholds of 20 %, 10 %, and 5 %, respectively. The prevalence of mutations according to drug class and treatment status is shown in Fig. 1. The prevalence of mutations increased with decreasing NGS cut-off thresholds across all drug classes in both treatment-

Table 1
Demographic and viro-immunological features according to ART status (treatment naïve vs experienced PLHIV) at the time of genotypic resistance testing.

Features	All PLHIV (N = 121)	Treatment Naïve (N = 85)	Experienced (N = 36)
Sex, n (%)			
Male	89 (74 %)	70 (82 %)	19 (53 %)
Female	32 (26 %)	15 (18 %)	17 (47 %)
Born in Italy, n (%)			
Yes	94 (78 %)	67 (79 %)	27 (75 %)
No	27 (22 %)	18 (21 %)	9 (25 %)
Age, years, median (IQR)	46 (37–53)	43 (34–53)	48 (44–54)
CD4 T cell count, cell/μL, median (IQR)[a]	124 (50–346)	150 (44–353)	82 (57–311)
HIV viral load, copies/ml,[b] median (IQR)	125,000 (17,600–787,000)	257,500 (1700–934,500)	19,050 (2547–225,750)

[a] Available for 120 PLHIV (85 ART-naive; 35 ART-experienced).
[b] Available for 120 PLHIV (84 ART-naïve; 36 ART-experienced).

Table 2
HIV-1 subtypes according to Country of Origin (Italian-born vs non-Italian PLHIV) and treatment status (naïve vs experienced), and univariate analysis.

HIV Subtype	All PLHIV (N = 120[a])	Italian-born PLHIV (N = 94)	Non-Italian PLHIV (N = 26)	P_1	Naïve (N = 84[a])	Experienced (N = 36)	P_2
B	65 (54 %)	62 (66 %)	3 (11 %)	**<0.001**	40 (48 %)	25 (69 %)	**0.024**
A1	7 (6 %)	4 (4 %)	3 (11 %)	0.185	5 (6 %)	2 (5 %)	0.944
C	4 (3 %)	4 (4 %)	0 (0 %)	0.574	3 (3 %)	1 (3 %)	1.000
F1	8 (7 %)	7 (7 %)	1 (4 %)	0.682	7 (8 %)	1 (3 %)	0.433
G	2 (2 %)	1 (1 %)	1 (4 %)	0.398	2 (2 %)	0	1.000
CRFs	34 (28 %)	16 (17 %)	18 (69 %)	**<0.001**	27 (32 %)	7 (19 %)	0.191

[a] HIV-1 subtype was not determined in 1 non-Italian treatment naive person. CRFs: circulating recombinant forms.

Table 3
High-level resistance at 20 % NGS cut-offs to the main antiviral molecules used in the clinical practice by absolute and relative frequencies.

Drug	Overall population N = 121	Treatment-naïve N = 85	Treatment-experienced N = 36
Tenofovir	1 (0.8 %)	0 (0 %)	1 (2.8 %)
Lamivudine	5 (4.13 %)	0 (0 %)	5 (13.9 %)
Abacavir	0 (0 %)	0 (0 %)	0 (0 %)
Emtricitabine	0 (0 %)	0 (0 %)	0 (0 %)
Rilpivirine	0 (0 %)	0 (0 %)	0 (0 %)
Doravirine	1 (0.8 %)	1 (1.2 %)	0 (0 %)
Etravirine	0 (0 %)	0 (0 %)	0 (0 %)
Efavirenz	2 (1.6 %)	2 (2.3 %)	0 (0 %)
Darunavir	0 (0 %)	0 (0 %)	0 (0 %)
Raltegravir	5 (4.1 %)	3 (3.5 %)	2 (5.5 %)
Cabotegravir	3 (2.5 %)	1 (1.2 %)	2 (5.5 %)
Dolutegravir	0 (0 %)	0 (0 %)	0 (0 %)
Bictegravir	0 (0 %)	0 (0 %)	0 (0 %)

naïve and experienced PLHIV. Not all mutations were associated with drug resistance.

The detection of drug resistance increased with decreasing NGS cut-off thresholds. Specifically, with an NGS cut-off of 20 %, drug resistance was found in 22 cases (18.2 %), with resistance affecting one class in all but one test. Of these cases, 12 were treatment-naïve PLHIV (14.1 %), while 10 were treatment-experienced PLHIV (27.8 %), $P = 0.088$. With 10 % and 5 % NGS cut-offs, the prevalence of resistance reached 25.6 % and 33.0 %, respectively. The prevalence of resistance by drug class and treatment status is depicted in Fig. 1.

Absolute frequencies of mutations according to NGS cut-offs are reported in Fig. 2.

High-level resistance to antiretroviral drugs at 20 % NGS cut-offs is represented in Table 3.

The lowering of NGS cut-offs mainly increased the rates of INSTIs resistance. For treatment-naïve PLHIV, resistance to raltegravir increased to 5 cases (5.9 %) with a 10 % cut-off and to 12 cases (14.1 %) with a 5 % cut-off; resistance to cabotegravir increased to 4 cases (4.7 %) with a 5 % cut-off, and resistance to bictegravir and dolutegravir appeared in 2 cases (2.3 %) with a 5 % cut-off. For treatment-experienced individuals, resistance to lamivudine increased to 6 cases (16.7 %) with a 5 % cut-off, and resistance to raltegravir increased to 3 cases (8.3 %) with a 10 % cut-off.

3.4. Prevalence of mutations and resistance at 20 %, 10 %, and 5 % ngs frequency cut-offs according to genotype

For overall resistance, no difference was observed between B and non-B subtypes for any NGS cut-offs (20 %: $P = 0.390$; 10 %: $P = 0.574$; 5 %: $P = 0.850$). For overall mutation prevalence, a significant difference was reported at 10 % NGS cut-off (55.4% vs. 33.9 %, $P = 0.018$). No differences were observed for the 20 % ($P = 0.116$) and 5 % ($P = 0.063$) NGS cut-offs. Differences in mutations and resistances for specific drug classes are reported in Table 4. Notably, the mutation T215 was found only in the B sub-

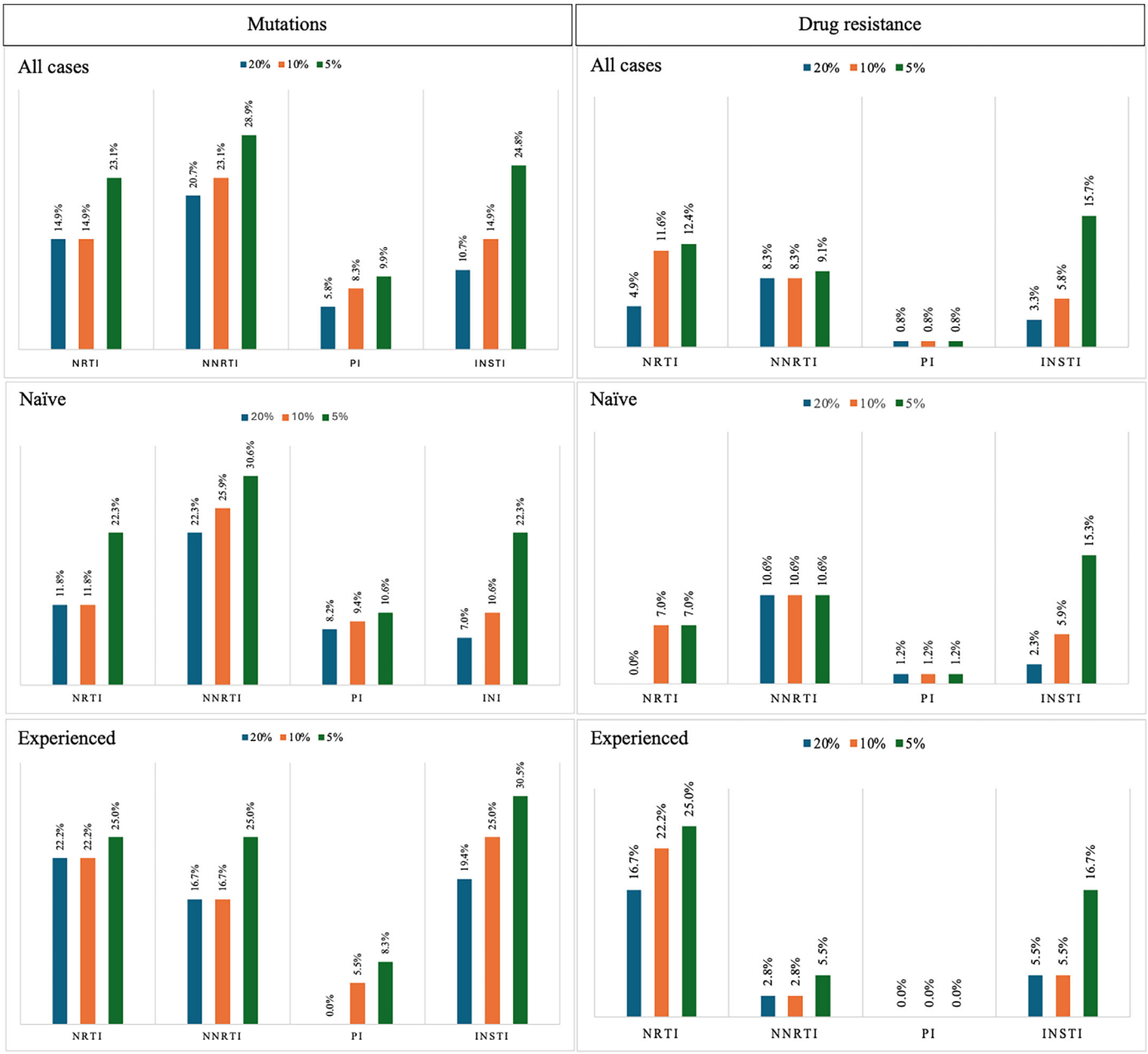

Fig. 1. Prevalence of mutations according to drug class and treatment status detected at different NGS cut-offs (20 %, 10 %, 5 %) (on the left); prevalence of resistance detected at different NGS cut-offs (20 %, 10 %, 5 %) according to drug class and treatment status (on the right).

type (9.2% vs. 0.0 %, $P = 0.020$, all NGS cut-offs), and Y143H was found in non-B subtypes (10.7% vs. 0.0 %, $P = 0.007$, 5 % NGS cut-off).

4. Discussion

Our study describes the prevalence of HIV-1 mutations and drug resistance in a cohort of PLHIV presenting at the University Hospital in Palermo, using the NGS method for the analysis and applying three different NGS frequency cut-offs of ≥5 %, ≥10 %, and ≥20 %. In Italy, previous studies have reported the prevalence of drug resistance and mutations using the Sanger method [9–23], and the main studies are summarized in Table 5. Italian studies on HIV-1 subtypes and prevalence of drug resistance are heterogeneous, often involving multicenter cohorts where overlapping data may occur between different studies. The most employed method for GRT was Sanger sequencing, revealing a low prevalence of mutations for PIs and INSTIs in treatment-naïve individuals. At the same time, higher rates of mutations were observed for reverse transcriptase inhibitors. However, INSTI resistance analysis was reported in a few studies [9,15,17–20,22]. In a previous Italian study, Fabeni et al. showed an overall prevalence of drug resistance of 14.3 % ($n = 501$), mainly involving NNRTI (9.6 %) in a large multicenter cohort of treatment-naïve PLHIV [13]. More recently, Fabeni et al. analyzed a large cohort of PLHIV in north and central Italy between the years 2015 and 2021, showing a transmitted drug resistance prevalence to any class of 8.0 % by Sanger technology, constant over time [23]. This study, however, did not report data on PLHIV from South Italy and Sicily. In our study, using a Sanger-like 20 % NGS cut-off, an overall prevalence of mutations of 39.7 % was observed. Most of these mutations were not associated with HIV-1 drug resistance, which was ascertained in

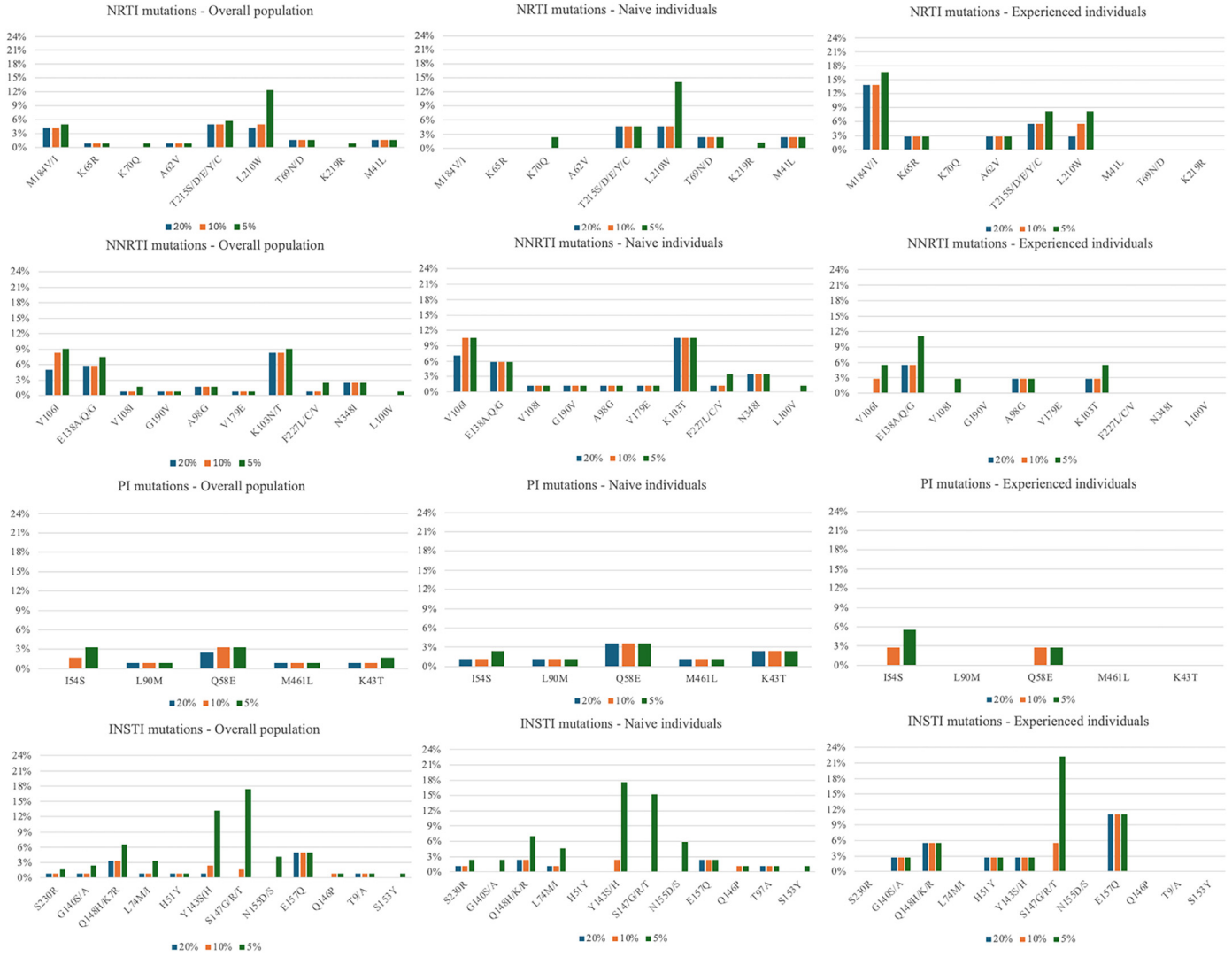

Fig. 2. Frequencies of single mutations according to drug class and treatment status.

18.2 % of GRTs. Distinguishing by ART status, the prevalence of HIV-1 drug resistance was 14.1 % for treatment-naïve and 27.8 % for treatment-experienced individuals. The prevalence of overall drug resistance in naïve individuals was consistent with previous Italian studies, where it ranged between 7.9 % and 14.3 %. For treatment-experienced PLHIV, a previous cohort described by Maggiorella et al. [16] reported a slightly lower value of 23.4 % compared to our result (27.8 %). Reducing the NGS cut-off to 5 % increased the prevalence of mutations and drug resistance to 53.7 % and 33.0 %, respectively. Scant data are available in the literature on the effects of using NGS with different thresholds for HIV GRT. The START trial, one of the largest global cohorts with NGS characterization of transmitted drug resistance in treatment-naïve individuals, showed that the overall prevalence rates of transmitted drug resistance using NGS cut-offs of 2 %/5 %/20 % were 9.2 %/5.6 %/3.2 % for NRTIs, 9.2 %/6.6 %/4.9 % for NNRTIs, 11.4 %/5.5 %/2.4 % for PIs, and 3.5 %/1.6 %/0.1 % for INSTIs [24]. Comparatively, in our treatment-naïve population, the prevalence of drug resistance was higher for all classes except PIs for the corresponding NGS thresholds. In our analysis, the INSTI class was most affected by the reduction of the cut-off due to the appearance of additional mutations and resistance, both in treatment-naïve and experienced individuals, though with a significantly higher prevalence in the latter. These mutations had little impact on the new INSTIs dolutegravir and bictegravir, with resistance emerging in only 2 cases at a 5 % cut-off, while at the same lower cut-off resistance to cabotegravir, currently used in long-acting therapy, was found in 6 cases. Resistance to raltegravir increased from 4.1 % to 12.4 % using a 5 % NGS cut-off. A lower prevalence of INSTI resistance was highlighted in a previous study, which observed no major INSTI resistance-associated mutations in a cohort of treatment-naïve PLHIV between 2014 and 2016 in the United Kingdom, confining INSTI resistance only to low-frequency variants [25]. We found that the increase in drug resistance with decreasing NGS cut-offs was less evident for other drug classes. For NRTIs, treatment-experienced individuals showed a higher prevalence of resistance at all NGS cut-offs, with the prevalence increasing to 25.0 % at a 5 % threshold, while in treatment-naïve individuals, it was 7.0 % at the same cut-off. For NRTIs, M184V/I was the most common drug resistance mutation, observed only in treatment-experienced PLHIV. This mutation is known to be detected in PLHIV treated with lamivudine, conferring resistance to lamivudine and emtricitabine and reduced susceptibility to abacavir, although it causes reduced viral fitness [26]. NNRTIs and PIs showed lower differences in the prevalence of resistance with changing cut-offs, though the prevalence of mutations increased in both treatment-naïve and experienced PLHIV. There is no strong evidence that mutations affecting minority variants can influence therapeutic choice, except for some studies sug-

Table 4
Analysis of demographic and laboratory data and mutations and drug resistance rates according to subtype B and non-B.

Variables	B (N = 65)	Non-B (N = 56)	*P*
Age, years, median (IQR)	47 (39–54)	44.5 (34–52.25)	0.268
CD4 T cell count, cell/μL, median (IQR)[a]	116 (54–392)	124 (34–315.5)	0.297
HIV viral load, copies/ml, median (IQR)[b]	73,300 (11,700–284,769)	385,000 (59,950–1,150,000)	**< 0.001**
Sex Male	51 (78.4 %)	38 (67.8 %)	0.187
Mutations NRTI			
20 %	15 (23.1 %)	3 (5.35 %)	***P* = 0.009**
10 %	15 (23.1 %)	3 (5.35 %)	***P* = 0.009**
5 %	19 (29.2 %)	9 (16.1 %)	*P* = 0.129
Resistance NRTI			
20 %	5 (7.7 %)	1 (1.8 %)	*P* = 0.215
10 %	12 (18.5 %)	2 (3.6 %)	***P* = 0.011**
5 %	13 (20.0 %)	2 (3.6 %)	***P* = 0.011**
Mutations NNRTI			
20 %	14 (21.5 %)	11 (19.6 %)	*P* = 0.797
10 %	17 (26.1 %)	11 (19.6 %)	*P* = 0.397
5 %	22 (33.8 %)	13 (23.2 %)	*P* = 0.198
Resistance NNRTI			
20 %	3 (4.6 %)	7 (12.5 %)	*P* = 0.185
10 %	3 (4.6 %)	7 (12.5 %)	*P* = 0.185
5 %	4 (6.1 %)	7 (12.5 %)	*P* = 0.343
Mutations PI			
20 %	5 (7.7 %)	2 (3.6 %)	*P* = 0.449
10 %	7 (10.8 %)	3 (5.3 %)	*P* = 0.337
5 %	9 ((13.8 %)	3 (5.3 %)	*P* = 0.139
Resistance PI			
20 %	0 (0.0 %)	1 (1.8 %)	*P* = 0.463
10 %	0 (0.0 %)	1 (1.8 %)	*P* = 0.463
5 %	0 (0.0 %)	1 (1.8 %)	*P* = 0.463
Mutations INSTI			
20 %	5 (7.7 %)	8 (14.2 %)	*P* = 0.243
10 %	8 (12.3 %)	10 (17.8 %)	*P* = 0.392
5 %	14 (21.5 %)	16 (28.57 %)	*P* = 0.372
Resistance INSTI			
20 %	2 (3.1 %)	2 (3.6 %)	*P* = 1.000
10 %	3 (4.6 %)	4 (7.1 %)	*P* = 0.703
5 %	9 (13.8 %)	10 (17.8 %)	*P* = 0.545

[a] Available for 120 PLHIV (85 ART-naive; 35 ART-experienced).
[b] Available for 120 PLHIV (84 ART-naïve; 36 ART-experienced).

gesting an impact on NNRTI-based regimens [27,28]. Furthermore, it is important to distinguish the technical cut-off from the clinical cut-off. The technical cut-off provides information about the reliability of results obtained from genotypic resistance tests, whereas the clinical cut-off identifies which mutated variants should be considered when selecting antiretroviral therapy. Reducing the NGS cut-off could also introduce technical errors due to stop codons, unusual mutations, and APOBEC sequences [29]. In the present study, we wanted to evaluate the prevalence of HIV-1 minority variants at NGS cut-off set at 5 %, according to the manufacturer's recommendations. On the other hand, a recent study conducted by the Italian HIV-1 NGS network and the ARCA Cohort, involving 11 virology laboratories, demonstrated that with the routine method for NGS-GRT (commercial kit AD4SEQ HIV-1 Solution v2, Arrow Diagnostics S.r.l., Genoa, Italy), only mutations with a frequency >10 % were reliably detected across different NGS data processing tools [30]. In fact, most discordant mutations were generally observed at frequencies below 10 % [30]. Therefore, according to these findings, our results on NGS cut-off set at 5 % should be interpreted with caution. It should be highlighted that the NGS cut-off at which results can be considered reliable may depend on the NGS method used. Indeed, for example, in another study conducted by the Italian PRESTIGIO group, HIV-1 DNA NGS analysis with a technical cut-off set at 5 % was shown to be acceptable when using a homemade Nextera-based library preparation method for genotypic HIV testing. This study focused on a cohort of non-viremic individuals harboring multidrug-resistant virus strains. They also showed that the number of major drug resistance mutations was significantly higher in individuals who later experienced virological rebound compared to those who maintained virological control [31]. Our study highlighted several HIV-1 subtypes in our cohort, finding that subtype B was the most common, though non-B subtypes were observed in 46 % of the population. Among them, CRFs, primarily CRF02_AG, were the most reported, involving mainly non-Italian individuals but also affecting Italians, demonstrating a significant circulation of non-B subtypes in Italy. These data are consistent with previous Italian [9–20,23] and European [32,33] studies, which have highlighted a growing incidence of non-B HIV-1 subtypes. A study by the ICONA Foundation showed that HIV-1 clustering transmission among newly diagnosed PLHIV in Italy is primarily driven by natives, mainly men who have sex with men with recent diagnoses, and they are frequently infected with non-B subtypes [13]. A previous study in Northern Italy identified subtype F1 and CRF02_AG in 17 % and 51 % of 710 naïve PLHIV, respectively, and this result was correlated to increasing migratory flows [14]. In our study, overall mutation prevalence was increased in subtype B when using a 10 % NGS cut-off. A previous Italian study using the Sanger sequencing method observed that the detection rate of drug resistance in patients infected with subtype B was higher than in those infected with non-B subtypes (11.6% vs. 6 %; $P = 0.03$) [15]. Our analysis showed a significantly higher prevalence of NRTI mutations with 20 % and 10 % NGS cut-offs, while resistance was significantly higher with 10 % and 5 % NGS cut-offs. The mutation T215Y/F, which

Table 5
Major previous Italian studies on HIV-1 drug resistance among treatment-naïve and treatment-experienced PLHIV.

Authors and years	Population	Subtypes	Sequencing method	INSTI analysis (yes or not)	Drug resistance (at least one)	Main mutations
Muccini et al., 2023 [9].	1223 ART-naïve PLHIV between 2009 and 2019, single-center study.	B 79.4 % (78.0 % were Italians), CRF01_AE (more frequent non-B subtype 74.0 %).	Sanger using an ABI PRISM 3130xl Genetic Analyzer.	Yes.	Overall not reported; PI 1.8 %; NRTI 4.2 %; NNRTI 3.8 %; INSTI 1.5 % (only reduced susceptibility to INSTI).	PI: not reported. NRTI: not reported. NNRTI: not reported. INSTI: T97A, L74I, E157Q, G163R.
Fabeni et al.,2021 [10].	229 ART-naïve PLHIV, between 2013 and 2020, single-center study.	B 56.8 %, F1 9.2 %, CRF02_AG 7.9 %, C 6.6 %, A1 5.2 %, CRF12_BF (3.9 %).	Sanger.	Not.	Overall 10.0 %; PI 3.9 %; NRTI 1.3 %; NNRTI 5.2 %.	Not reported.
Bozzi et al., 2021 [11].	186 ART-naïve PLHIV, between 2008 and 2014, multicenter study.	Non-B 32.3 % (CRF02_AG 11.3 %, F1 9.1 %, C3.8 %, CRF18_cpx 1.6 %, others 6.5 %).	Sanger.	Not.	Overall 8.6 %; PI 1.6 %; NRTI 4.8 %; NNRTI 2.7 %.	Not reported.
Bavaro et all, 2020 [12].	1155 ART-naïve non-Italy born PLHIV from the ARCA database between 1998 and 2017.	Non-B 61.7 %.	Not reported.	Not.	Overall 8.6 %; PI 2.1 %; NRTI 3.9 %; NNRTI 4.3 %.	PI: M46I, L90M. NRTI: M41L, M184V, T215D. NNRTI: K103N.
Fabeni et al., 2020 [13].	3499 ART-naïve PLHIV from the ICONA cohort between 1998 and 2018.	B 73.1 %, CRF02_AG 5.3 %, F1 5.1 %, C 4.2 %, A1 3.0 %, CRF60_BC 51.8 %, other CRFs 7.5 %.	Sanger.	Not.	Overall 14.3 %; PI 1.9 %; NRTI 4.3 %; NNRTI 9.6 %.	PI: K23I, M46I/L, F53Y, I85V, L90M. NRTI: M41L, D67N, L210W, T215S/L/D, K219Q. NNRTI: L100I, K101E, K103N/S, V106I, E138A/G/K, G190A.
Lorenzin et al., 2020 [14].	710 ART-naïve PLHIV, between 2011 and 2017, single-center study.	Non-B 43 % (54 % Italians), (CRF02_AG, 51.2 %, F1 17 %, C 9.2 %, G 6.1 %, CRF01_AE 5.8 %, CRF06_cpx 4 %, CRF12_BF 1.7 %, CRF09_cpx 1.7 %).	Sanger, (TruGene Siemens Healthcare Diagnostics GmbH, Eschborn, Germany).	Not.	Not reported.	PI: V82I, M46L, and I54V. NRTI: V106I, M41L, and T215D/S. NNRTI: K103N and K101Q/R.
Mazzuti et al., 2020 [15].	668 ART-naïve PLHIV, between 2006 and 2017, single-center study.	Non-B 32.9 %, 22.7 % of Italians and 55.7 % of non-Italians (CRF02_AG 8.4 %, and subtypes C and F both 6.0 %).	Sanger, TruGene® HIV-1 Genotyping Kit (Siemens Healthcare Diagnostics Inc., Deerfield, IL, USA).	Yes, (52 PLHIV).	Overall 9.4 %; PI 1 %; NRTI 4.2 %; NNRTI 5.8 %; INSTI 3.8 % (2/52).	PI: M46I and I54M/L/V. NRTI: T215D/S, D67N and M41L. NNRTI: K103N, E138G/K/Q, V108I and Y181. INSTI: Q148H and G140
Maggiorella et al., 2020 [16].	192 non-Italy born PLHIV (88 % experienced) from the Italian Network for HIV Characterization between 2008 and 2017.	B 23.4 %, A 9.4 %, C 10.4 %, D 1.6 %, F 5.2 %, G 16.1 %, probable CRF 33.9 %.	Sanger.	Not.	Overall 23.4 %.	PI: M46I. NRTI: M184V. NNRTI: K103N.
Calza et al., 2020 [17].	178 ART-naïve PLHIV between 2017 and 2019, single-center study.	B 57 %.	Sanger sequencing approach (Viroseq, Abbott Diagnostics, Abbott Park Road, IL).	Yes.	Overall 7.9 %; PI 2.8 %; NRTI 3.9 %; NNRTI 3.4 %; INSTI 0.9 %.	PI: F53Y, M46I/L. NRTI: T215S/I/D, M41L, D67N/G, L210W, K219Q. NNRTI: K103N. INSTI: E138K.
Santoro et al., 2020 [18].	17 highly treatment-experienced PLHIV who failed an INSTI-based regimen from the PRESTIGIO registry.	B 94.1 %.	Sanger, GeneSeqIN.	Yes.	Data about PI, NRTI, and NNRTI regarded previous samples. INSTI 82.3 %.	INSTI: E138A/K, G140S, Y143C/H/R, Q148H, N155H.
Modica et al., 2019 [19].	462 INSTI-experienced PLHIV (356 'INSTI-failing) from the ARCA database between 2008 and 2017.	B 70.6 %, CRF02_AG 6.1 % (more frequent non-B subtype).	Sanger, Viroseq HIV-1 Genotyping System.	Yes.	INSTI 40 % (at least low-level resistance 42.9 %).	INSTI: N155H, Q148H/K/R, G140A/C/S, E138A/K/T and Y143C/H/R.
Ungaro et al., 2019 [20].	94 Italian ART-experienced perinatally infected PLHIV from the ARCA database.	Not reported.	Not reported.	Yes.	Overall 85 %; PI 35 %; NRTI 79 %; NNRTI 65 %; INSTI 7 %.	PI: M46I/L. NRTI: M184V/I. NNRTI: K103N/S. INSTI: Q148H.
Franzetti et al., 2018 [21].	2155 ART-naïve (435 non-Italy born) PLHIV from the ICONA cohort between 2007 and 2014.	Not reported.	Not reported.	Not.	Overall 10.7 %; PI 2.2 %; NRTI 6.0 %; NNRTI 4.0 %.	Not reported.

(*continued on next page*)

Table 5 (*continued*)

Authors and years	Population	Subtypes	Sequencing method	INSTI analysis (yes or not)	Drug resistance (at least one)	Main mutations
Montagna et al., 2015 [22].	1730 genotype tests (46.4 % ART-naïve) from 1402 PLHIV, between 2003 and 2014, single-center study.	Not reported.	Sanger, TruGene HIV-1 Genotyping Kit (Siemens Healthcare Diagnostics Inc., Deerfield, IL).	Yes.	Over the 2003–2014, resistance to NRTIs, NNRTIs, and PIs declined from 80.0 % to 18.7 %, from 42.8 % to 20.1 % and from 74.2 % to 8.3 %, respectively. INSTI 4.7 %.	PI: L33F, M46I/L, G48V, I54V, V82A, L90M. NRTI: M184V, M41L, L210W. NNRTI: K101E, K103N, G190A/S. INSTI: N155H, Y143R.
Fabeni et al., 2024 [23].	2386 ART-naïve (1341 Italians) between 2015 and 2021, multicenter study.	B 55.4 %	Sanger.	Yes (1831 PLHIV).	Overall 8.0 %, PI 1.3 % NRTI 2.6 % NNRTI 4.8 % INSTI 0.3 %	PI: I85V, M46L, NRTI: M41L, T215S, L210W, M184V NNRTI: K103N, K101E, G190A, V106I, E138A. INSTI: E138K

PI, protease inhibitor, NRTI, nucleos(t)ide reverse transcriptase inhibitor; NNRTI, non-nucleoside reverse transcriptase inhibitor; INSTI, integrase strand transfer inhibitor.

causes intermediate/high-level resistance to zidovudine and potentially low-level resistance to abacavir and tenofovir, was found only in subtype B, while Y143H, which causes resistance to raltegravir, was found in non-B subtypes.

5. Conclusion

Our study confirms a significant circulation of non-B subtypes among Italian PLHIV that had not been previously detected in Sicily. Using the NGS method, we observed an increase in mutations and resistance with the reduction of the cut-off level, especially involving the INSTIs. The prevalence of resistance for at least one drug in this class was 3.3 %, 5.8 %, and 15.7 %, with cut-offs of 20 %, 10 %, and 5 %, respectively. The mutations detected mainly affected first-generation INSTIs, while dolutegravir and bictegravir confirmed their high genetic barrier. Treatment-experienced individuals showed a higher prevalence of resistance to NRTIs. PIs showed the lowest rates of resistance, with 0 % in treatment-experienced and 1.2 % in treatment-naive individuals. Resistance to NNRTIs was little affected by changing the NGS cut-off.

6. Contributors

LP wrote the original draft, collected the data, and contributed to the analysis, MT collected the data, CM collected the data and contributed to the analysis, SC collected the data and contributed to the analysis, RG collected the data, IG collected the data, GD collected the data, BR collected the data, MMS conceptualized this study and revised the paper, GMG conceptualized this study and revised the paper, AC conceptualized this study, supervised the study and revised the paper, CB conceptualized this study, performed genotyping resistance tests and revised the paper. All the authors approved the final version of the manuscript.

Funding

None.

Ethical statement

This study was approved by the Ethics Committee “Palermo I”, Palermo, Italy (Verbal n.17 04/07/2024).

Declaration of competing interest

None declared.

References

[1] https://www.who.int/data/gho/data/themes/hiv-aids accessed 18.12.23.
[2] Hemelaar J. Implications of HIV diversity for the HIV-1 pandemic. J Infect 2013;66(5):391–400. doi:10.1016/j.jinf.2012.10.026.
[3] Aldrich C, Hemelaar J. Global HIV-1 diversity surveillance. Trends Mol Med 2012;18(12):691–4. doi:10.1016/j.molmed.2012.06.004.
[4] https://www.eacsociety.org/media/guidelines-12.0.pdf accessed 18.12.23
[5] Ávila-Ríos S, Parkin N, Swanstrom R, et al. Next-generation sequencing for HIV drug resistance testing: laboratory, clinical, and implementation considerations. Viruses 2020;12(6):617. doi:10.3390/v12060617.
[6] Behjati S, Tarpey PS. What is next generation sequencing? Arch Dis Child Educ Pract editor 2013;98(6):236–8. doi:10.1136/archdischild-2013-304340.
[7] Lee ER, Gao F, Sandstrom P, Ji H. External quality assessment for next-generation sequencing-based hiv drug resistance testing: unique requirements and challenges. Viruses 2020;12(5):550. doi:10.3390/v12050550.
[8] http://hivdb.stanford.edu/ accessed 07.02.24
[9] Muccini C, Galli L, Sampaolo M, Gianotti N, Castagna A, Canetti D. Increasing trend of transmitted integrase inhibitor resistance in a cohort of antiretroviral therapy-naive people living with HIV. J Antimicrob Chemother 2023;78(5):1314–15. doi:10.1093/jac/dkad109.
[10] Fabeni L, Rozera G, Berno G, et al. Molecular transmission dynamics of primary HIV infections in Lazio Region, years 2013-2020. Viruses 2021;13(2):176. doi:10.3390/v13020176.
[11] Bozzi G, Fabeni L, Abbate I, et al. Non-B subtypes account for a large proportion of clustered primary HIV-1 infections in Italy. Sex Transm Infect 2023;99(1):53–6. doi:10.1136/sextrans-2021-055289.
[12] Bavaro DF, Di Carlo D, Rossetti B, et al. Pretreatment HIV drug resistance and treatment failure in non-Italian HIV-1-infected patients enrolled in ARCA. Antivir Ther 2020;25(2):61–71. doi:10.3851/IMP3349.
[13] Fabeni L, Santoro MM, Lorenzini P, et al. Evaluation of HIV transmission clusters among natives and foreigners living in Italy. Viruses 2020;12(8):791. doi:10.3390/v12080791.
[14] Lorenzin G, Gargiulo F, Caruso A, et al. Prevalence of non-B HIV-1 subtypes in North Italy and analysis of transmission clusters based on sequence data analysis. Microorganisms 2019;8(1):36. doi:10.3390/microorganisms8010036.
[15] Mazzuti L, Melengu T, Falasca F, et al. Transmitted drug resistance mutations and trends of HIV-1 subtypes in treatment-naïve patients: a single-centre experience. J Glob Antimicrob Resist 2020;20:298–303. doi:10.1016/j.jgar.2019.08.024.
[16] Maggiorella MT, Sanarico N, Brindicci G, et al. High HIV-1 diversity in immigrants resident in Italy (2008-2017). Sci Rep 2020;10(1):3226. doi:10.1038/s41598-020-59084-2.
[17] Calza L, Tamburello M, Borderi M, et al. Prevalence of transmitted drug resistance mutations among newly diagnosed HIV-1-infected patients in a large teaching hospital of the Northern Italy. J Med Virol 2020;92(8):929–31. doi:10.1002/jmv.25896.
[18] Santoro MM, Fornabaio C, Malena M, et al. Susceptibility to HIV-1 integrase strand transfer inhibitors (INSTIs) in highly treatment-experienced patients who failed an INSTI-based regimen. Int J Antimicrob Agents 2020;56(1):106027. doi:10.1016/j.ijantimicag.2020.106027.
[19] Modica S, Rossetti B, Lombardi F, et al. Prevalence and determinants of resistance mutations in HIV-1-infected patients exposed to integrase inhibitors in a large Italian cohort. HIV Med 2019;20(2):137–46. doi:10.1111/hiv.12692.
[20] Ungaro R, Taramasso L, Bruzzone B, et al. Prevalence of acquired resistance mutations in a large cohort of perinatally infected HIV-1 patients. Clin Microbiol Infect 2019;25(11):1443–6. doi:10.1016/j.cmi.2019.07.004.
[21] Franzetti M, De Luca A, Ceccherini-Silberstein F, et al. Evolution of HIV-1 transmitted drug resistance in Italy in the 2007-2014 period: a weighted analysis. J Clin Virol 2018;106:49–52. doi:10.1016/j.jcv.2018.07.009.
[22] Montagna C, Mazzuti L, Falasca F, et al. Trends in drug resistance-associated

mutations in a real-life cohort of Italian patients infected with HIV-1. J Glob Antimicrob Resist 2015;3(4):267–72. doi:10.1016/j.jgar.2015.07.006.
[23] Fabeni L, Armenia D, Abbate I, et al. HIV-1 transmitted drug resistance in newly diagnosed individuals in Italy over the period 2015-21. J Antimicrob Chemother 2024;79(9):2152–62. doi:10.1093/jac/dkae189.
[24] Baxter JD, Dunn D, Tostevin A, et al. Transmitted HIV-1 drug resistance in a large international cohort using next-generation sequencing: results from the strategic timing of antiretroviral treatment (START) study. HIV Med 2021;22(5):360–71. doi:10.1111/hiv.13038.
[25] Mbisa JL, Ledesma J, Kirwan P, et al. Surveillance of HIV-1 transmitted integrase strand transfer inhibitor resistance in the UK. J Antimicrob Chemother 2020;75(11):3311–18. doi:10.1093/jac/dkaa309.
[26] van de Klundert MAA, Antonova A, Di Teodoro G, et al. Molecular epidemiology of HIV-1 in Eastern Europe and Russia. Viruses 2022;14(10):2099. doi:10.3390/v14102099.
[27] Stella-Ascariz N, Arribas JR, Paredes R, Li JZ. The role of HIV-1 drug-resistant minority variants in treatment failure. J Infect Dis 2017;216(suppl_9):S847–50. doi:10.1093/infdis/jix430.
[28] Cozzi-Lepri A, Dunn D, Tostevin A, et al. Rate of response to initial antiretroviral therapy according to level of pre-existing HIV-1 drug resistance detected by next-generation sequencing in the strategic timing of antiretroviral treatment (START) study. HIV Med 2024;25(2):212–22. doi:10.1111/hiv.13556.
[29] Manyana S, Gounder L, Pillay M, Manasa J, Naidoo K, Chimukangara B. HIV-1 drug resistance genotyping in resource limited settings: current and future perspectives in sequencing technologies. Viruses 2021;13(6):1125. doi:10.3390/v13061125.
[30] Armenia D, Carioti L, Micheli V, et al. Comparison of different HIV-1 resistance interpretation tools for next-generation sequencing in Italy. Viruses 2024;16(9):1422. doi:10.3390/v16091422.
[31] Armenia D, Spagnuolo V, Bellocchi MC, et al. Use of next-generation sequencing on HIV-1 DNA to assess archived resistance in highly treatment-experienced people with multidrug-resistant HIV under virological control: data from the PRESTIGIO registry. J Antimicrob Chemother 2024;79(9):2354–63. doi:10.1093/jac/dkae236.
[32] Marcelin AG, Grude M, Charpentier C, et al. Resistance to integrase inhibitors: a national study in HIV-1-infected treatment-naive and -experienced patients. J Antimicrob Chemother 2019;74(5):1368–75. doi:10.1093/jac/dkz021.
[33] Hanke K, Faria NR, Kühnert D, et al. Reconstruction of the genetic history and the current spread of HIV-1 subtype A in germany. J Virol 2019;93(12):e02238-18. doi:10.1128/JVI.02238-18.