Journal of Medical Virology

WILEY

JOURNAL OF MEDICAL VIROLOGY

RESEARCH ARTICLE OPEN ACCESS

Development of a Next-Generation Sequencing Protocol for Assessing Lenacapavir Resistance in HIV-1 Capsid

Omar El Khalili[1,2] | Collins Ambe Chenwi[1,2,3] | Daniele Spalletta[1,2] | Greta Marchegiani[1,2] | Luca Carioti[1] | Hossein Eizadi Moghadam[1] | Ada Bertoli[1,4] | Vincenzo Spagnuolo[5] | Maria Mercedes Santoro[1] | Francesca Ceccherini-Silberstein[1] | Maria Concetta Bellocchi[1]

[1]Department of Experimental Medicine, University of Rome Tor Vergata, Rome, Italy | [2]Ph.D. Course in Microbiology, Immunology, Infectious Diseases, and Transplants (MIMIT), University of Rome Tor Vergata, Rome, Italy | [3]Chantal Biya International Reference Centre for Research on HIV and AIDS Prevention and Management (CIRCB) | [4]Virology Unit, Tor Vergata University Hospital, Rome, Italy | [5]Infectious Diseases Unit, San Raffaele Scientific Institute, Milan, Italy

Correspondence: Maria Concetta Bellocchi (bellocchi@med.uniroma2.it)

Received: 12 June 2025 | **Revised:** 3 November 2025 | **Accepted:** 8 December 2025

Funding: Gilead Sciences; Innovative Health Initiative Joint Undertaking (IHI JU), Grant/Award Number: 101194735

Keywords: Capsid | HIV-1 | lenacapavir | next-generation sequencing | resistance

ABSTRACT

Lenacapavir (LEN) is a first-in-class capsid inhibitor (CAI) that targets multiple stages of the HIV-1 lifecycle, showing efficacy in heavily treatment-experienced (HTE) individuals with multidrug-resistance (MDR) and in pre-exposure prophylaxis (PrEP). This study aimed to characterize a novel in-house next-generation sequencing (NGS) protocol targeting HIV-1 capsid (CA) region using both HIV-1 RNA from plasma and HIV-1 DNA from peripheral-blood-mononuclear-cells (PBMCs). A total of 60 samples (41 HIV-1 RNA and 19 HIV-1 DNA) with various HIV-1 subtypes and viremia levels were tested. Overall, molecular amplification was successful in 83.3% of cases (75.6% for HIV-1 RNA and 100% for HIV-1 DNA), while high quality sequences were obtained in 76.7% of samples (65.9% for HIV-1 RNA and 100% for HIV-1 DNA). Among RNA samples with viremia ≥ 500 copies/mL, sequencing success reached 92.6%, showing a statistically significant association with viral load. Subtype-specific analysis showed amplification and sequencing rates of 86.0% and 79.1% for subtype B, and 76.5% and 70.6% for non-B subtypes, with no significant difference. Reproducibility was fully confirmed by pairwise similarity analyses at 10% and 20% frequency cutoff, upon reprocessing 13 HIV-1 RNA samples. This protocol provides an important tool, primarily for subtype B, for personalized HIV-1 treatment with CAI-based strategies, enabling efficient characterization of LEN resistance mutations in the CA region, using both DNA and RNA samples.

1 | Introduction

Despite the progress made in improving the efficacy and safety of antiretroviral therapy (ART), HIV-1 cure is not feasible and therefore continues to be a major global public health issue [1]. HIV-1 infection remains a serious concern in a proportion of heavily treatment experienced (HTE) people with HIV-1 (PWH) who harbor a multidrug-resistance (MDR) virus, often as a result of exposure to suboptimal treatment and multiple treatment failures [2]. For this fragile population ART options can also be limited.

In this context, strategies for prevention of new infections and treatment are fundamental for reducing the individual and

Omar El Khalili and Collins Ambe Chenwi Equally contribution to this work as first author.

Francesca Ceccherini-Silberstein and Maria Concetta Bellocchi Equally contribution to this work as last author.

societal burden of HIV-1. In this light, a continuous development of new agents active against resistant variants of HIV-1 and targeting novel mechanisms of action is required, in order to provide simpler and more efficacious treatment options to all PWH, irrespective of their prior treatment history. Among new antiretrovirals, lenacapavir (LEN) is a novel injectable first-in-class HIV-1 capsid inhibitor (CAI). It inhibits selectively multiple stages of capsid function, by directly binding to the interface between the capsid protein subunits, preventing the nuclear import of proviral DNA, hindering virus assembly and release by interfering with the function of *Gag/Gag-pol* genes, and thereby generating malformed capsids [3]. LEN use is indicated, in combination with other antiretrovirals, for the treatment of HTE individuals with MDR and has recently shown high efficacy for prevention (more than 99%) when used as pre-exposure prophylaxis (PrEP) in diverse populations (cisgender women, cisgender gay, bisexual, and other men, transgender women, transgender men, and gender-nonbinary persons) [4–6].

Integrating into clinical routine new pharmacological targets, such as the capsid, is crucial for optimizing treatment strategies and improving clinical outcomes. Due to the novelty of this target, a method for characterizing the resistance associated mutations in the capsid is mandatory. Margot et al. in a recent study used the Sanger sequencing method to evaluate the capsid resistance mutations in 27 HIV-1 RNA plasma samples [7]. However, there are limitations of Sanger sequencing in detecting low-frequency variants and providing adequate resistance profiles [8]. This has led to the need for the implementation of the genotypic test for the capsid, by using next-generation sequencing (NGS) technologies, which offers greater sensitivity and accuracy in identifying resistance-associated mutations. The advent of NGS has redefined genome sequencing techniques. This technology sequences millions of fragments simultaneously per run, enabling the detection of minority quasispecies with mutations occurring at frequencies below 20%, with an accuracy greater than 99%. Evidence suggests that some mutations present in HIV-1 resistant minority variants may be clinically relevant [9, 10].

The aim of this study was to develop a novel NGS protocol to sequence HIV-1 capsid region for assessing resistance to LEN by using both HIV-1 RNA from plasma samples and HIV-1 DNA from peripheral blood mononuclear cells (PBMCs) matrices, with diverse subtypes and viremia levels.

2 | Materials and Methods

2.1 | Clinical Samples

For the protocol development, residual anonymized specimens were used, originating from routine clinical practice and/or research activities within two cohorts of PWH living in Italy: the PRESTIGIO Registry (https://trials-ice2.advicepharma.com/PRESTIGIO/) and the ICONA Foundation (https://www.fondazioneicona.org/). Ethic Committee approval was deemed unnecessary under Italian law for residual anonymized samples used for diagnostic purposes since this was not considered a clinical trial of medicinal products for clinical use (Art. 6 and Art. 9, Law Decree 211/2003). For residual anonymized samples obtained from research activities, approval was obtained by the Ethic Committee of each participating center involved in the above-mentioned Italian cohorts.

2.2 | HIV-1 Extraction and Amplification

Viral RNA was extracted from 1 mL of plasma using the QIAamp Viral RNA Mini Kit (Qiagen GmbH, Hilden, Germany), after ultracentrifugation at 23,000 g for 2 h at 4°C, following the manufacturer's instructions, while DNA was extracted from a pellet of 5×10^6 PBMCs using the High Pure PCR Template Preparation Kit (Roche Diagnostics, Basel, Switzerland).

HIV-1 RNA from plasma and HIV-1 DNA from PBMCs were subjected to reverse transcription/amplification and amplification, respectively, using the SuperScript III One-Step RT-PCR system for long templates (Invitrogen, Carlsbad, CA, USA). In detail, each 50 μL reaction contained: 10 μL of extracted viral genome, 25 μL of 2× reaction mix, 8 μL of $MgSO_4$ (5 mmol/L), 3 μL of DNase/RNase-free water, 0.75 μL each of forward and reverse primers (10 pmol/μL), 1 μL of RNase Out (40 U/μL; replaced with 1 μL of water for HIV-1 DNA samples) and 1.5 μL of RT/Taq enzyme. Thermal cycler conditions were 50°C for 30 min, 94°C for 2 min, followed by 45 cycles of 95°C for 30 s, 53°C for 30 s, and 72°C for 2 min; final extension at 72°C for 10 min. When no visible amplification product was detected after agarose gel electrophoresis of the first-round PCR, a nested PCR was performed. The 50 μL reaction mix contained: 5 μL of first-round PCR product, 33 μL of DNase/RNase-free water, 5 μL of PCR buffer (10×), 3.5 μL of $MgCl_2$ (25 mM), 1 μL of dNTPs (10 mmol/L), 0.9 μL each of forward and reverse primers (10 pmol/μL) and 0.7 μL of AmpliTaq Gold DNA polymerase (Life Technologies, Carlsbad CA, USA). Thermal cycler conditions were 93°C for 12 min, 40 cycles of 95°C for 30 s, 56°C for 30 s, and 72°C for 2 min, with a final extension at 72°C for 10 min. Amplicons were analyzed by agarose gel electrophoresis to confirm band sizes (See Figure 1). *Gag* specific primers and thermal profiles were adapted from Soria et al. [11] (See Table 1).

2.3 | HIV-1 Next Generation Sequencing

Each amplified sample was purified using (0.8× ratio) Ampure XP Beads (Beckman Coulter, Pasadena, CA, USA), then quantified

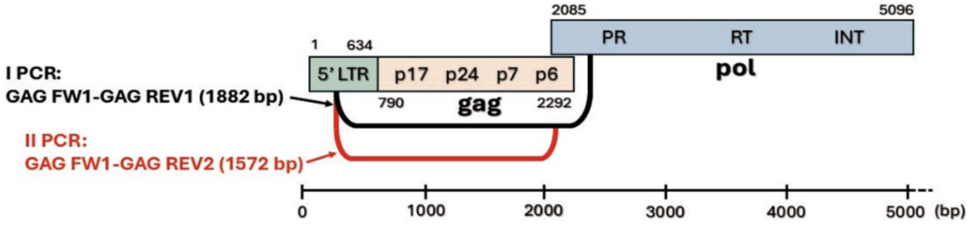

FIGURE 1 | Mapping of HIV-1 capsid amplification regions.

TABLE 1 | Primer sequence and thermal profiles for amplification.

Gene	Primer[a]	Sequence (5′−3′)	Position[b]	Thermal profile	Amplification
Gag	*Gag* FW1	GCCTCAATAAAGCTTGCCTT	522–541	1 cycle 50°C, 30 min[c] 1 cycle to 94° C for 2 min 45 cycles (95°C 30 s, 53°C 30 s, 72°C 2 min) 1 cycle at 72°C 10 min	I RT/PCR
	Gag REV1	CCAATTCCCCCTATCATTTTT	2384–2404		
	Gag FW1	GCCTCAATAAAGCTTGCCTT	522–541	1 cycle to 93° C for 12 min 40 cycles (95°C 30 s, 56°C 30 s, 72°C 2 min) 1 cycle at 72°C 10 min	II PCR Nested
	Gag REV2	CCCTAAAAAATTAGCCTGTCT	2074–2094		

[a]FW: Sense primer, REV: Antisense primer.
[b]Positions according to HXB2 (Accession number: K03455.1) Numbering System.
[c]Removed in case of HIV-1 DNA specimens. Min: minutes; sec: seconds.

using Qubit dsDNA HS Assay Kit (Invitrogen, Carlsbad, CA, USA) with Qubit 2.0 Fluorometer (Life Technologies, Carlsbad, CA, USA). For each sample, 1 ng of amplicon was involved in a tagmentation reaction by Nextera XT DNA Library Kit (Illumina Inc., San Diego, CA, USA) and uniquely indexed with Nextera XT Index Kit v2 (Illumina Inc., San Diego, CA, USA) following the manufacturer's instructions. After a second purification (0.6x ratio) and second quantification, the libraries were diluted at 4 nM and pooled. Finally, 15 pM of the denatured pool was sequenced paired-end with MiSeq Reagent Kits v2 (2 × 250) (Illumina Inc., San Diego, CA, USA) with 6%–10% of PhiX Control V3 library to monitor sequencing quality [12].

2.4 | Bioinformatics Analyses, Mutational Pattern and Subtype Assignment

NGS data obtained as FASTQ files were analyzed using HIVdb Stanford algorithm (version 9.8; https://hivdb.stanford.edu/) to determine the mutational pattern of each sample. Sequences were considered valid with at least 100 coverage reads per position, based on commonly used parameters in the literature [13–15]. The consensus sequences for all samples were generated with prevalence cutoffs of 5%, 10%, and 20% mutation detection threshold (MDT) as defined by the Stanford HIDVB website (https://hivdb.stanford.edu/hivdb-capsid/by-reads/). The HIV-1 subtype was assessed using two automated tools, COMET (https://comet.lih.lu/) and Stanford (https://hivdb.stanford.edu/) and the results for each sample were compared with those obtained from a previous subtyping analysis on protease/reverse transcriptase. In addition, a quality control of the raw data obtained in the FASTQ format was performed with Trimmomatic [16] software in order to remove adapters, PCR primers and poor-quality reads. FASTQ files were analyzed with VirVarSeq software version 1 [17] using K03455.1 as reference sequence. This biostatistical analysis was performed using a higher coverage threshold of 1,000, to increase the robustness of variant detection for each sample; a consensus sequence with prevalence cutoff of 5%, 10% and 20% was generated using quasitools (https://github.com/phac-nml/quasitools).

2.5 | Efficiency Assessment

The efficiency assessment of the protocol was conducted, considering the main variables that could influence the success rate: the different compartment (HIV-1 RNA from plasma and HIV-1 DNA from PBMCs), the HIV-1 subtype (B *vs*. non-B), and viremia levels for the HIV-1 RNA samples. In particular, viremia levels were stratified according to the following strata: ≥ 50–500; ≥ 500–1000; ≥ 1000–10,000; ≥ 10,000 copies/mL. The efficiency of the two main steps of the process (molecular amplification and sequencing on the Miseq platform) were considered separately.

2.6 | Reproducibility Assessment

For both precision and reproducibility testing, the variability in the result is determined based on nucleotide sequence similarity by comparison with the consensus sequences derived from the replicates (at 5%, 10%, and 20% cutoffs, as described before.) Briefly, sequences were aligned with the HXB2 reference for *Gag* and CA regions using MAFFT v7.475. The CA region (positions 397–1089 of *Gag* or 1186–1878 relative to HXB2, resulting in 693 nucleotides and 231 residues) was manually extracted with BioEdit v7.7. Pairwise sequence similarity for each sample across two runs was assessed using the Needleman-Wunsch algorithm implemented in EMBOSS Needle [18]. Acceptance criteria was more than 90% of pairwise comparisons with at least 98% identical (with non-matching mixtures counted as a difference) [19].

2.7 | Statistical Analysis

Descriptive statistics were expressed as median values and the interquartile range (IQR) for continuous variables and the number (percentage) for categorical variables. To evaluate the impact of HIV-1 subtype (B vs. non-B) and viral load stratification (< 500 vs. ≥ 500 copies/mL) on amplification and sequencing success, the Fisher exact test was applied for categorical variables. All statistical analyses were performed using the SPSS software package for Windows (version 23.0, SPSS Inc., Chicago, IL). A two-sided *p*-value of less than 0.05 was considered statistically significant.

3 | Results

3.1 | Sample Characteristics

Sixty samples were analyzed, of which 41 (68.3%) were HIV-1 RNA, obtained from plasma samples, and 19 (31.7%) were

TABLE 2 | Sample characteristics.

Characteristics	HIV-1 RNA Plasma	HIV-1 DNA PBMCs[a]
Sample, *N* (%)	41 (68.3)	19 (31.7)
HIV-1 RNA, copies/mL, Median (IQR)	2014 (183–12,070)	/
HIV-1 RNA, copies/mL, Minimum	52	/
HIV-1 RNA, copies/mL, Maximum	9.4×10^6	/
Subtype B, *N* (%)[b]	25 (61.0)	18 (94.7)
HIV-1 RNA ranges, *N* (%)[b]		
≥ 50–1,000 copies/mL	18 (43.9)	/
a. ≥ 50–500 copies/mL	*14 (34.1)*	/
b. ≥ 500–1,000 copies/mL	*4 (9.8)*	/
≥ 1,000–10,000 copies/mL	13 (31.7)	/
≥ 10,000 copies/mL	10 (24.4)	/

[a]Even PBMC samples were obtained from viremic individuals and 12 during virological suppression.
[b]Percentages refer to subgroup (41 HIV-1 RNA and 19 HIV-1 DNA). IQR: interquartile range; PBMCs: peripheral-blood-mononuclear-cells.

HIV-1 DNA, extracted from PBMCs. Specifically, 42 PWH were included in the study: 32 had only HIV-1 RNA samples, 2 had only HIV-1 DNA samples (each with two HIV-1 DNA samples), and 8 individuals had both HIV-1 RNA and HIV-1 DNA samples, with HIV-1 DNA samples collected at different time points. Among these 8 individuals: 3 had one HIV-1 RNA and two HIV-1 DNA samples, 3 had one HIV-1 RNA and one HIV-1 DNA sample, 1 had two HIV-1 RNA and two HIV-1 DNA samples, and 1 had one HIV-1 RNA and four HIV-1 DNA samples. All plasma samples had detectable viremia, with a median [IQR] value of 2014 [183–12,070] HIV-1 RNA copies/mL. Among plasma samples, 18 (43.9%) had HIV-1 RNA levels in the range ≥ 50–1000 copies/mL (≥ 50–500 copies/mL: 14 samples; ≥ 500–1000 copies/mL: 4 samples), 13 (31.7%) had HIV-1 RNA levels in the range ≥ 1000-10,000 copies/mL, and 10 (24.4%) presented HIV-1 RNA levels ≥ 10,000 copies/mL (See Table 2). All HIV-1 DNA samples were collected from 10 individuals receiving LEN-based antiretroviral therapy at different time points. Seven PBMC samples were obtained from viremic individuals (with a median HIV-RNA of 23,554 [1447-32,200] copies/ml), while 12 were collected during virological suppression (plasma HIV-RNA ≤ 50 copies/ml).

Overall, HIV-1 subtype B was detected in 43 samples (71.7%), of which 25 were HIV-1 RNA samples and 18 were HIV-1 DNA samples. Non-B subtypes were identified in the remaining 17 samples (28.3%), predominantly among HIV-1 RNA ($n = 16$) and in only one HIV-1 DNA sample (see Table 2). The non-B subtypes included 4 samples with CRF02_AG (6.7%), 3 with subtype F1 (5.0%), 2 with the DF recombinant form (3.3%, one in the HIV-1 DNA sample), 2 with subtype C (3.3%), and 1 with subtype G (1.7%). The remaining 5 samples (8.3%) harbored other recombinant forms: CRF09_cpx ($n = 1$), CRF12_BF ($n = 1$), CRF41_CD ($n = 1$), CRF42_BF ($n = 1$), and CRF60_BC ($n = 1$).

3.2 | Efficiency of the Developed NGS Protocol

Overall, high-quality CA region sequences were obtained from 46 out of 60 samples, corresponding to a global sequencing success rate of 76.7%, with 1600 minimum reads per position and 100% *gag* coverage found. The main factors potentially affecting the success rate of the developed NGS protocol were carefully evaluated. These included the viral genome specimen (HIV-1 RNA vs. HIV-1 DNA), the HIV-1 subtype, and the level of viremia in HIV-1 RNA samples.

The analysis was performed by evaluating amplification efficiency of the CA region, sequencing efficiency among successfully amplified samples, and the overall sequencing success rate across all samples. The results are summarized in Table 3.

3.2.1 | Efficiency by Sample Compartment: HIV-1 RNA and HIV-1 DNA Samples

The first variable evaluated to assess the efficiency of the developed NGS protocol was the viral genome compartment, considering different sample types: HIV-1 RNA from plasma sample and HIV-1 DNA extracted from PBMCs.

Considering the compartment, successful sequences were obtained from 27 of 41 HIV-1 RNA samples (65.9%) and in all 19 HIV-1 DNA samples (100%) (see Table 3).

In terms of amplification efficiency, the CA region was successfully amplified in 31 of 41 HIV-1 RNA samples (75.6%), and in all 19 HIV-1 DNA samples (100%). Among the 31 amplified RNA samples, 27 (87.1%) yielded analyzable sequences, while 4 (12.9%) failed sequencing due to low coverage (< 100 reads).

3.2.2 | Efficiency by HIV-1 Subtype-Associated Variability: B Subtype versus Non-B Subtypes

The second variable analyzed was the impact of HIV-1 subtype-associated viral variability on the success rate. Specifically, samples with different non-B subtypes were merged, and efficiency was evaluated by comparing the B subtype versus non-B subtypes ($N = 43$ vs. $N = 17$). For B subtype plasma samples, the median [IQR] viremia was 1726 [171–14,415] copies/mL, while for non-B subtype samples, the median viral load was 2788 [185–15,030] copies/mL.

Regarding sequencing success in the overall study population, 34 out of 43 samples with HIV-1 subtype B (79.1%) and 12 out of 17 samples with HIV-1 non-B subtypes (70.6%) yielded valid sequences. In particular, among the 43 HIV-1 B subtype

TABLE 3 | Amplification and sequencing performance on HIV-1 RNA and HIV-1 DNA.

		Amplification success	NGS success	Overall success
HIV-1 RNA ($N = 41$)	Overall, N (%)	31 (75.6)	27/31 (87.1)	27/41 (65.9)
		By HIV-1 subtype		
	B, $N = 25$	19 (76.0)	16/19 (84.2)	16/25 (64.0)
	Non-B, $N = 16$	12 (75.0)	11/12 (91.7)	11/16 (68.8)
		By HIV-1 RNA ranges		
	≥ 50–1000 copies/ml, $N = 18$	9 (50.0)	5/9 (55.6)	5/18 (27.8)
	a. ≥ 50–500 copies/mL, $N = 14$	6 (42.9)	2/6 (33.3)	2/14 (14.3)
	b. ≥ 500–1000 copies/mL, $N = 4$	3 (75.0)	3/3 (100)	3/4 (75.0)
	≥ 1000–10,000 copies/mL $N = 13$	12 (92.3)	12/12 (100)	12/13 (92.3)
	≥ 10,000 copies/mL, $N = 10$	10 (100)	10/10 (100)	10/10 (100)
HIV-1 DNA ($N = 19$)	Overall, N (%)	19 (100)	19/19 (100)	19/19 (100)
		By HIV-1 subtype		
	B, $N = 18$	18 (100)	18/18 (100)	18/18 (100)
	Non-B, $N = 1$	1 (100)	1/1 (100)	1/1 (100)

Abbreviations: NGS, next generation sequencing.

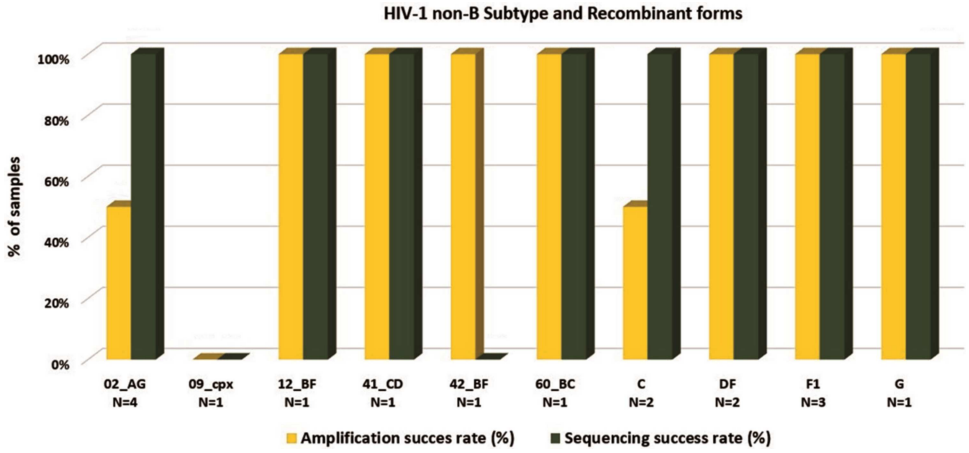

FIGURE 2 | Amplification and sequencing performance among HIV-1 non-B subtypes ($N = 17$). The figure shows (a) the amplification success rates (yellow bars) calculated as the percentage of successfully amplified samples within each subtype group; (b) the sequencing success rates (green bars), calculated as the percentage of samples, exclusively among those successfully amplified. Overall, four non-B subtype samples failed amplification: two CRF02_AG samples (HIV-1 RNA, 192 copies/mL and 222 copies/mL, respectively), one 09_cpx (HIV-1 RNA, 183 copies/mL) and one subtype C (HIV-1 RNA, 101 copies/mL). Only one successfully amplified sample did not yield a valid sequence after sequencing (42_BF; HIV-1 RNA, 81 copies/mL). The only non-B HIV-1 DNA sample (DF) was successfully amplified and sequenced.

samples, amplification was successful in 37 (86.0%; 19 HIV-1 RNA, 18 HIV-1 DNA) samples. For the 17 non-B subtype samples, 13 (76.5%; 12 HIV-1 RNA, 1 HIV-1 DNA) samples were successfully amplified.

Regarding the sequencing success according to positive amplification: 34/37 (91.9%) subtype B samples yielded valid sequences (16 HIV-1 RNA, 18 HIV-1 DNA), whereas 12/13 (92.3%) non-B samples generated valid sequences (11 HIV-1 RNA, 1 HIV-1 DNA).

A Fisher's exact test comparing samples with HIV-1 subtype B ($n = 43$) and non-B subtypes ($n = 17$) showed no statistically significant difference in amplification success rates (B = 37/43; non-B = 13/17; $p = 0.448$), and in the overall sequencing success (B = 34/43; non-B = 12/17; $p = 0.511$). Thus, HIV-1 subtype variability did not appear to affect the performance.

Figure 2 shows amplification success rates calculated within each HIV-1 non-B subtype and sequencing success rates among those successfully amplified.

3.2.3 | *Efficiency by Viremia Levels of HIV-1 RNA Samples*

Efficiency was additionally assessed by categorizing the 41 HIV-1 RNA plasma samples according to viral load ranges.

Amplification and sequencing success were both 100% (10/10) in the ≥ 10,000 copies/mL range and 92.3% (12/13) in the ≥ 1000–10,000 copies/mL range. To further investigate performance at lower viral loads, a sub-analysis focusing on samples with viral loads below 1000 copies/mL was conducted. In the ≥ 500–1000 copies/mL range, both amplification and sequencing success were 75.0% (3/4). In the ≥ 50–500 copies/

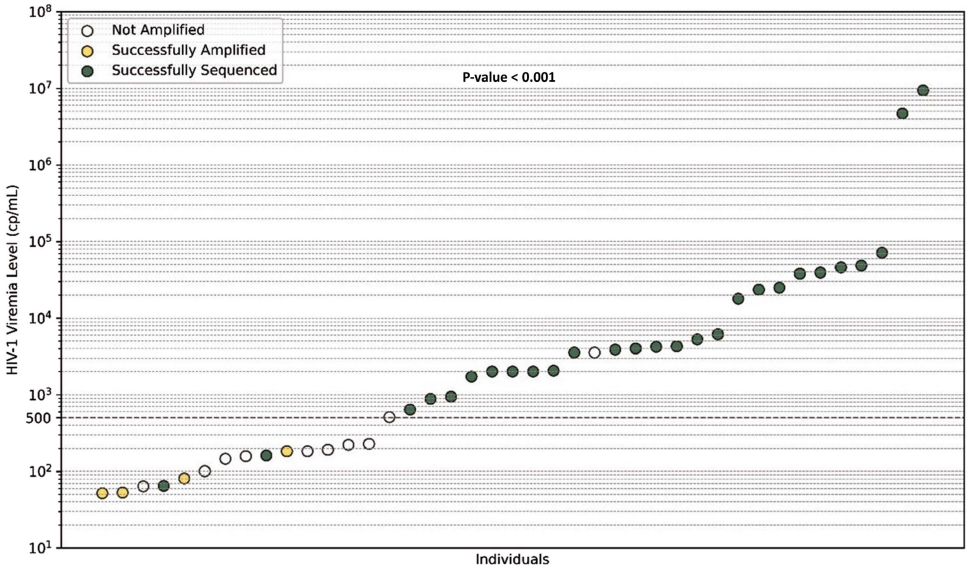

FIGURE 3 | Amplification and sequencing performance by HIV-1 RNA viremia ranges. The figure shows the relationship between viremia levels and performance (amplification and sequencing success). Each dot represents an individual, yellow dots indicate successful amplification, green dots indicate successful amplification and sequencing. Failures are shown in white dots. The dashed line indicates the HIV-1 RNA threshold of 500 copies/mL. Only two amplification failures occurred in samples with viremia above 500 copies/mL (512 and 3,564 copies/mL), both with subtype B. By Fisher's exact test a significant association was observed for viral loads < 500 copies/mL ($n = 14$) compared to ≥ 500 copies/mL ($n = 27$), with both amplification and overall sequencing success showing $p < 0.001$.

mL range, amplification success decreased to 42.9% (6/14), and sequencing success was 14.3% (2/14), (see Table 3).

Notably, samples with viral loads above 500 copies/mL showed high reliability, with 25 out of 27 (92.6%) successfully amplified and sequenced.

Statistical analysis confirmed a significant association between viral load and both amplification and sequencing success (Fisher's exact test, both $p < 0.001$), as shown in Figure 3.

3.3 | Evaluation of NGS Reproducibility

To evaluate the reproducibility of the developed protocol, 13 representative HIV-1 RNA samples with successful amplification and analyzable sequences were reprocessed, starting from the same extraction sample. All reprocessed samples showed consistent performance, with successful amplification and sequencing achieved in every case. Using the pairwise similarity sequence test with EMBOSS Needle, nucleotide sequences obtained from the two processes were compared for each sample at NGS cut-offs of 5%, 10%, and 20% (see Table 4).

All samples demonstrated very high similarity values. Reproducibility acceptance criteria were met at all NGS analysis cut-offs, excluding the most stringent 5% threshold. The proportion of samples with at least 98% pairwise similarity was 100% (13/13) at both the 20% and 10% cut-offs. The lowest pairwise similarity observed was 96.7% at the 5% cut-off. The specific amino acid changes identified at the three NGS cut-offs (5%, 10%, and 20%) for each of the 13 sample pairs are detailed in Supplementary Table S1.

3.4 | LEN Resistance Profile

Overall, within the 46 CA sequences obtained, no major mutations associated with resistance to LEN were detected, except for two sequences derived from a single individual with a virological failure during LEN-based therapy. In this case, the resistance-associated mutation K70H (97.8%) and Q67K (98.5%) were identified in the HIV-1 RNA sequence, and were also confirmed in the corresponding proviral HIV-1 DNA at frequencies of 75.6% and 75.7%, respectively. This was the only individual among those who had both HIV-1 RNA and HIV-1 DNA samples who harbored LEN-resistance mutations.

Furthermore, the accessory mutation T107A was observed in three sequences from three distinct individuals, with variant frequencies of 98.2% and 66.9% in two HIV-1 RNA sequences, and 5.9% in a HIV-1 DNA sequence, respectively.

Finally, the accessory mutation A105T, at a frequency of 6.0%, was detected in only one sequence of the replicated HIV-1 RNA samples used to assess protocol reproducibility at 5% cutoff (see Table S1).

4 | Discussion

In this study, a novel and highly effective NGS protocol was developed for sequencing the HIV-1 CA region to assess resistance to LEN, a recently approved CA inhibitor for antiviral treatment, with significant potential applicability to both current and future antiretroviral drugs targeting this protein. The previously published protocol based on Sanger sequencing (Soria et al., 2016) was modified and optimized for the Illumina NGS platform to enable efficient amplification and deep sequencing of the HIV-1 CA region across various sample matrices (HIV-1 DNA and HIV-1 RNA), different subtypes, and viremia levels. The method is versatile and robust and can be implemented in both diagnostic and research settings, particularly for assessing LEN resistance in HTE PWH with MDR, either at baseline before initiating LEN therapy or in cases of virological failure [20]. To date, the novelty of this protocol lies in its ability to detect low-

TABLE 4 | Capsid NGS reproducibility performance on 13 repeated HIV-1 RNA samples.

Sample ID	Viral clade	HIV-1 RNA copies/mL	Nucleotide similarity 5%	Percentage similarity 5%	Nucleotide similarity 10%	Percentage similarity 10%	Nucleotide similarity 20%	Percentage similarity 20%	CAI mutations*
NGS-CA_R01	B	48,700	684	98.7%	688	99.3%	693	100%	None
NGS-CA_R02	B	468,1314	688	99.3%	691	99.7%	692	99.9%	None
NGS-CA_R03	B	38,084	688	99.3%	691	99.7%	692	99.9%	None
NGS-CA_R04	60_BC	3900	691	99.7%	691	99.7%	691	99.7%	One accessory
NGS-CA_R05	B	4239	692	99.9%	692	99.9%	692	99.9%	None
NGS-CA_R06	41_CD	6151	683	98.6%	689	99.4%	692	99.9%	None
NGS-CA_R07	B	5276	670	96.7%	679	98.0%	689	99.4%	None
NGS-CA_R08	B	1726	691	99.7%	691	99.7%	693	100%	None
NGS-CA_R09	12_BF	3561	677	97.7%	681	98.3%	686	99.0%	None
NGS-CA_R10	B	4021	690	99.6%	690	99.6%	690	99.6%	None
NGS-CA_R11	02_AG	2014	691	99.4%	692	99.9%	692	99.9%	None
NGS-CA_R12	B	884	691	99.7%	690	99.6%	690	99.6%	None
NGS-CA_R13	B	642	676	97.5%	682	98.4%	688	99.3%	None

Note: Pairwise nucleotide similarity across runs for each sample was evaluated in the CA region of the *gag* gene (693 nt) at 5%, 10%, and 20% NGS thresholds, as defined by the Stanford HIVdb (https://hivdb.stanford.edu/). Results are shown as percentages.

*CAI mutations: amino acid changes associated with resistance to LEN were assessed according to the major and accessory mutations listed in Stanford Algorithm v9.8. Only one accessory mutation (A105T) was found at 5% cutoff.

frequency variants through the implementation of an NGS-based approach, and to efficiently analyze both HIV-1 RNA samples and HIV-1 DNA derived from PBMCs.

The protocol achieved a 100% success rate with HIV-1 DNA samples and about 66% with HIV-1 RNA samples. Amplification failures were predominantly observed in HIV-1 RNA samples with low-level viremia, particularly within the ≥ 50–500 copies/mL range, where a marked reduction in both amplification and sequencing success rates was observed. Accordingly, to better characterize performance in this critical range, a sub-analysis was conducted on samples with viral loads below 1000 copies/mL. Amplification and sequencing success were substantially decreased in the ≥ 50–500 copies/mL range, with only 2 out of 14 samples (14.3%) successfully amplified and sequenced. Conversely, performance improved considerably in the ≥ 500–1000 copies/mL range, where 3 out of 4 samples (75.0%) yielded successful results. Notably, a statistically significant association was found between viral load ≥ 500 copies/mL and sequencing success, with 92.6% of these samples successfully amplified and sequenced. This highlights the utility of the protocol, especially considering that most commercial kits are not validated for viral loads below 1,000 copies/mL.

The higher success rate observed with HIV-1 DNA samples can likely be attributed to the type of starting material. In the study, proviral DNA was extracted from PBMCs, resulting in higher nucleic acid yield and purity compared to whole blood. This excellent performance will enable genotypic resistance testing (GRT) in the HIV-1 DNA context, for individuals with undetectable or with low-level viremia.

Regarding the subtype issue, similar success rates were observed for both HIV-1 B and non-B subtypes (79.1% and 70.6%, respectively), with no statistically significant difference. In the study population, this was likely due to the consistent binding efficiency of the primers within the viral *gag* region. Amplification success appeared to be influenced more by other factors, particularly low-level viremia (< 500 copies/mL), which substantially reduced amplification efficiency.

Concerning the resistance, the population included also samples from individuals treated with LEN. One of them, who experienced a virologic failure, showed the major resistance mutation K70H with the Q67K at high prevalence (> 75%), in both HIV-1 RNA and HIV-1 DNA matrices. Furthermore, the accessory mutation T107A was identified in three distinct individuals: two in HIV-1 RNA samples (at 98.2% and 66.9% prevalence, respectively) and one in a HIV-1 DNA sample (5.9% prevalence). These results show that the protocol can effectively detect resistance-associated mutations, highlighting its relevance for monitoring the effectiveness of LEN and other future CA inhibitors. Notably, even low-prevalence mutations were identified, although their clinical significance remains to be established.

No recurrent mutations were observed in specific regions of the capsid across the study population. The distribution of differences appeared random, with no indication of sequencing bias. The observed mutational patterns were largely attributable to subtype variability relative to the reference sequence used for alignment.

Finally, to evaluate the reproducibility and sensitivity of the protocol, 13 representative HIV-1 RNA samples with different subtypes and viral loads were reprocessed. The amplification and sequencing results were confirmed. Using pairwise similarity comparison, all sequences at 10% and 20% frequency cutoff achieved the 100% criteria of reproducibility (≥ 98% similarity) [18]. While 76.9% reproducibility was achieved at the 5% threshold (with 10 out of 13 pairs showing ≥ 98% similarity). This result is likely due to technical limitations, and is in line with other NGS protocols, since a 10% frequency threshold is currently recommended for NGS-based GRT analyses [15].

A limitation of this study is the relatively small sample size (60 samples), particularly regarding the distribution of non-B subtypes in HIV-1 DNA samples. Although 28.3% of the study population carried non-B subtypes, only one HIV-1 DNA sample with a non-B subtype was available for analysis. This could limit the interpretation of results obtained from HIV-1 DNA samples, particularly with respect to non-B subtypes. Overall, no statistically significant differences were observed between B and non-B subtypes in terms of amplification or sequencing success, suggesting that subtype variability did not substantially impact assay performance. However, the small number of non-B samples may have reduced the statistical power of the analysis. Therefore, further studies with larger and more balanced subtype representation are warranted. Indeed, the subtypes analyzed were limited to the most common ones circulating in Italy [21]. Even if, non-B subtypes are increasing among newly diagnosed cases in Italy, LEN is primarily used in patients with long treatment histories and multidrug resistance, the majority of whom are infected with subtype B. Therefore, this protocol is particularly applicable to this setting and other European settings with similar HIV-1 molecular epidemiology.

It should be highlighted that all the amplification failures in samples with non-B subtypes occurred with HIV-1 RNA levels below 500 copies/mL. Additional data from non-B subtype samples with higher viral loads would be useful to further assess protocol performance. Having access to a larger number of non-B samples, especially those that failed amplification with low viremia (such as CRF02_AG or subtype C), would have the opportunity to better investigate whether the observed failures are attributable to low viral load, or if subtype-specific factors may also play a role. In cases of low-level viremia, increasing the input amount of viral RNA or modifying PCR cycling parameters may improve the performance.

Finally, regarding the HIV-1 DNA analysis, the use of PBMCs instead of whole blood could potentially limit the adoption of the protocol in diagnostic routine, as Ficoll separation involves additional steps, increased costs, and longer processing times.

5 | Conclusions

The study presents a novel highly effective NGS protocol for analyzing the HIV-1 CA region, viral target of LEN and other future CA inhibitors. The protocol achieved high success rates for both HIV-1 DNA and RNA samples with viremia levels ≥ 500 copies/ml. High amplification and sequencing success rates were achieved in HIV-1 subtype B samples, with similarly favorable outcomes observed in non-B subtypes, despite the limited number of cases. The overall reproducibility and robustness of the protocol remained very high, supporting its reliability and implementation in both clinical and research

applications particularly for the management of PWH receiving LEN, or future CA inhibitors, for treatment or HIV-1 prevention.

Author Contributions

Maria Concetta Bellocchi, Francesca Ceccherini-Silberstein, and Maria Mercedes Santoro conceived the project. Greta Marchegiani, Omar El Khalili, Ada Bertoli, Vincenzo Spagnuolo and Daniele Spalletta collected the samples and clinical and virological information. Greta Marchegiani, Daniele Spalletta and Omar El Khalili performed sequencing. Greta Marchegiani, Luca Carioti, Daniele Spalletta and Hossein Eizadi performed bioinformatic and statistical analyses. Omar El Khalili, Collins Ambes Chenwi, and Maria Concetta Bellocchi wrote the manuscript. Maria Mercedes Santoro and Francesca Ceccherini-Silberstein revised the manuscript. All authors read, revised and approved the final version of the manuscript.

Acknowledgments

Thanks are extended to all clinicians, virologists, statisticians, data managers, and the biological bank of the ICONA Foundation and the PRESTIGIO Registry. Additionally, gratitude is expressed to Ilaria Maugliani for data management of this study, and to Flavia Funari, Livia Benedetti, and Giulia Torre for their assistance in the laboratory experiments. Funding for this publication was partially provided by an unconditional grant by Gilead Sciences. The study was also supported by the Innovative Health Initiative Joint Undertaking (IHI JU) under grant agreement No 101194735. The JU receives support from the European Union's Horizon Europe research and innovation programme and COCIR, EFPIA, Europa Bio, MedTech Europe, Vaccines Europe, and Roboscreen.

ICONA Foundation Study Group

Board of directors: A d'Arminio Monforte (President), A. Antinori (Vice-President), S. Antinori, A. Castagna, R. Cauda, G. Di Perri, E. Girardi, R. Iardino, A. Lazzarin, G.C. Marchetti, C. Mussini, E. Quiros-Roldan, L. Sarmati, B. Suligoi, F. von Schloesser, P. Viale. **Scientific secretary:** A. d'Arminio Monforte, A. Antinori, A. Castagna, F. Ceccherini-Silberstein, A. Cingolani, A. Cozzi-Lepri, E. Girardi, A. Gori, S. Lo Caputo, G. Marchetti, F. Maggiolo, C. Mussini, M. Puoti, C.F. Perno. **Steering committee:** C. Agrati, A. Antinori, F. Bai, A. Bandera, S. Bonora, A. Calcagno, D. Canetti, A. Castagna, F. Ceccherini-Silberstein, A. Cervo, S. Cicalini, A. Cingolani, P. Cinque, A Cozzi-Lepri, A d'Arminio Monforte, A. Di Biagio, R. Gagliardini, A. Giacomelli, E Girardi, N Gianotti, A Gori, G Guaraldi, S. Lanini, G. Lapadula, M Lichtner, A Lai, S Lo Caputo, G Madeddu, F Maggiolo, V Malagnino, G Marchetti, C Mussini, S Nozza, CF Perno, S Piconi, C Pinnetti, M Puoti, E Quiros Roldan, R Rossotti, S Rusconi, MM Santoro, A Saracino, L Sarmati, V Spagnuolo, N Squillace, V Svicher, L Taramasso, A Vergori. **Statistical and monitoring team:** F Bovis, A Cozzi-Lepri, I Fanti, A Rodanò, M Ponzano, A Tavelli. **Community advisory board:** A Bove, M Cernuschi, L Cosmaro, M Errico, A Perziano, V Calvino. **Biological bank INMI and San Paolo:** S Carrara, S Graziano, G Prota, S Truffa, D Vincenti, Y D'Errico. **Participating physicians and centers:** Italy A Giacometti, A Costantini, V Barocci (Ancona); A Saracino, C Santoro, E Milano (Bari); F Maggiolo, C Suardi (Bergamo); P Viale, L Badia, S Cretella (Bologna); E Quiros Roldan, E Focà, C Minardi (Brescia); B Menzaghi, C Abeli (Busto Arsizio); L Chessa, F Pes (Cagliari); P Maggi, L Alessio (Caserta); B Cacopardo, B Celesia (Catania); J Vecchiet, K Falasca (Chieti); A Pan, S Dal Zoppo (Cremona); D Segala (Ferrara); F Vichi, MA Di Pietro (Firenze); T Santantonio, S Ferrara (Foggia); M Bassetti, E Pontali, S Blanchi, N Bobbio, G Mazzarello (Genova); M Lichtner, L Fondaco (Latina); S Piconi, C Molteni (Lecco); S Rusconi, G Canavesi (Legnano) A Chiodera, P Milini (Macerata); G Nunnari, G Pellicanò (Messina); A d'Arminio Monforte, S Antinori, A Lazzarin, G Rizzardini, M Puoti, A Gori, A Castagna, A Bandera, V Bono, MV Cossu, A Giacomelli, R Lolatto, MC Moioli, L Pezzati, C Tincati (Milano); C Mussini, C Puzzolante (Modena); P Bonfanti, G Lapadula (Monza); V Sangiovanni, I Gentile, V Esposito, FM Fusco, G Di Filippo, V Rizzo, N Sangiovanni (Napoli); AM Cattelan, S Marinello (Padova); A Cascio, C Colomba (Palermo); D Francisci, E Schiaroli (Perugia); G Parruti, F Sozio (Pescara); P Blanc, A Vivarelli (Pistoia); C Lazzaretti, R Corsini (Reggio Emilia); M Andreoni, A Antinori, R Cauda, C Mastroianni, A Cingolani, V Mazzotta, S Lamonica, M Capozzi, A Mondi, M Rivano Capparuccia, G Iaiani, C Stingone, L Gianserra, J Paulicelli, MM Plazzi, G d'Ettore, M Fusto (Roma); M Cecchetto, F Viviani (Rovigo); G Madeddu, A De Vito (Sassari); M Fabbiani, F Montagnani (Siena); A Franco, R Fontana Del Vecchio (Siracusa); BM Pasticci, C Di Giuli (Terni); GC Orofino, G Calleri, G Di Perri, S Bonora, G Accardo (Torino); C Tascini, A Londero (Udine); V Manfrin, G Battagin (Vicenza); G Starnini, A Ialungo (Viterbo).

PRESTIGIO Study Group

Steering Committee: Antonella Castagna (Coordinator), Vincenzo Spagnuolo (Operative Coordinator), Daniele Armenia, Stefano Bonora, Leonardo Calza, Anna Maria Cattelan, Giovanni Cenderello, Adriana Cervo, Laura Comi, Antonio Di Biagio, Emanuele Focà, Roberta Gagliardini, Andrea Giacomelli, Filippo Lagi, Giulia Marchetti, Stefano Rusconi, Francesco Saladini, Maria Santoro, Maurizio Zazzi.

Virology Team and Biological Bank: Andrea Galli, Daniele Armenia, Francesco Saladini, Maria Santoro, Maurizio Zazzi, BioRep SRL. **Study Coordinators:** Elisabetta Carini, Sabrina Bagaglio, Girolamo Piromalli. **Statistical and Monitoring Team:** Riccardo Lolatto, Nicolò Capra. **Enrolling Centers: ANCONA:** Marcello Tavio, Alessandra Mataloni Paggi; **AOSTA:** Silvia Magnani, Manuela Colafigli, **AVIANO:** Ornella Schioppa, Stefania Zanussi, Valentina Da Ros, Silvia Rossetto; **BARI:** Annalisa Saracino, Flavia Balena; **BERGAMO:** Laura Comi, Daniela Valenti; **BOLOGNA:** Pierluigi Viale, Leonardo Calza, Federica Malerba, Silvia Cretella, Riccardo Riccardi; **BRESCIA:** Francesco Castelli, Emanuele Focà, Davide Minisci; **BUSTO ARSIZIO:** Barbara Menzaghi, Maddalena Farinazzo, Chiara Abeli; **CATANIA:** Bruno Cacopardo, Maurizio Celesia, Michele Salvatore Paternò Raddusa, Carmen Giarratana; **CATANZARO:** Paolo Fusco, Vincenzo Olivadese, Simona Mongiardi; **CREMONA:** Angelo Pan, Chiara Fornabaio, Paola Brambilla; **FIRENZE:** Alessandro Bartoloni, Filippo Lagi, Paola Corsi, Trevisan Sasha, Gasparro Giuseppe, Cecilia Costa, Alessio Bellucci, Elisa Mariabelli; **FOGGIA:** Teresa Santantonio, Sergio Lo Caputo, Sergio Ferrara, Arianna Narducci; **GENOVA:** Emanuele Pontali, Marcello Feasi, Antonio Sarà, Matteo Bassetti, Antonio Di Biagio, Sabrina Blanchi; **LECCO:** Stefania Piconi, Martina Bottanelli, Silvia Pontiggia, Valsecchi Giada; **LEGNANO:** Stefano Rusconi, Cinzia Roberta Bassoli, Francesco Bassani, Liana Bevilacqua; **MILANO:** Antonella Castagna, Vincenzo Spagnuolo, Camilla Muccini, Elisabetta Carini, Sabrina Bagaglio, Riccardo Lolatto, Nicolò Capra, Andrea Galli, Rebecka Papaioannu, Tommaso Clemente, Golnaz Torkjazi, Girolamo Piromalli, Spinello Antinori, Andrea Giacomelli, Tiziana Formenti, Giulia Marchetti, Lidia Gazzola, Fabiana Trionfo Fineo, Massimo Puoti, Cristina Moioli, Federico D'Amico, Simoncini Elena, Sassi Serena; **MODENA:** Cristina Mussini, Adriana Cervo, Giulia Nardini; **NAPOLI:** Elio Manzillo, Antonella Gallicchio; **PADOVA:** Anna Maria Cattelan, Maria Mazzitelli; **PALERMO:** Antonio Cascio, Marcello Trizzino; **PARMA:** Elisa Fronti, Diletta Laccabue, Federica Carli; **PAVIA:** Roberto Gulminetti, Layla Pagnucco, Mattia Demitri, Alessandra Ferrari; **PERUGIA:** Daniela Francisci, Giuseppe De Socio, Elisabetta Schiaroli; REGGIO EMILIA: Elisa Garlassi, Romina Corsini; **ROMA:** Roberta Gagliardini, Marisa Fusto, Loredana Sarmati, Vincenzo Malagnino, Tiziana Mulas, Mirko Compagno, Carlo Torti, Simona Di Giambenedetto, Silvia Lamonica, Pierluigi Salvo; **SANREMO:** Giovanni

Cenderello, Rachele Pincino, Davide Laurenda; **SASSARI:** Giordano Madeddu, Andrea De Vito; **SIENA:** Mario Tumbarello, Massimiliano Fabbiani, Francesca Panza, Ilaria Rancan; **TORINO:** Giovanni Di Perri, Stefano Bonora, Micol Ferrara, Andrea Calcagno, Silvia Fantino, Giancarlo Orofino, Guido Calleri, Guastavigna Marta; **VERONA:** Stefano Nardi, Marta Fiscon.

Supported By: ViiV Healthcare, Gilead Sciences, MSD, Janssen-Cilag.

Ethics Statement

For this study, we used residual anonymized specimens from routine clinical practice and/or research activities within two cohorts of PWH living in Italy: the PRESTIGIO Registry (https://trials-ice2.advicepharma.com/PRESTIGIO/) and the ICONA foundation (https://www.fondazioneicona.org/). Approval by the Ethics Committee was deemed unnecessary under Italian law for residual anonymous samples evaluated for diagnostic purposes since this was not considered a clinical trial of medicinal products for clinical use (Art. 6 and Art. 9, Law Decree 211/2003). For residual samples obtained from research activities, approval was obtained by the Ethic Committee of all the participating centers involved in the above-mentioned Italian cohorts.

Conflicts of Interest

The authors declare no conflicts of interest.

Data Availability Statement

The data that support the findings of this study are available from the corresponding author upon reasonable request.

References

1. Joint United Nations Programme on HIV/AIDS (UNAIDS). 2024. The Urgency of Now: AIDS at a Crossroads. Geneva: Joint United Nations Programme on HIV/AIDS; 2024. Licence: CC BY-NC-SA 3.0 IGO.

2. M. B. Brizzi, T. L. Cable, D. C. Patel, K. Williams, Z. Adjei, and C. J. Fichtenbaum, "Heavily Treatment-Experienced Patients with HIV: Are New Mechanisms of Action Enough?," *Journal of International Medical Research* 52, no. 12 (2024), https://doi.org/10.1177/03000605241301883.

3. H. Dvory-Sobol, N. Shaik, C. Callebaut, and M. S. Rhee, "Lenacapavir: A First-in-Class HIV-1 Capsid Inhibitor," *Current Opinion in HIV and AIDS* 17, no. 1 (2022): 15–21, https://doi.org/10.1097/COH.0000000000000713.

4. V. Vardanega, E. New, D. Mezzio, and L. A. Eddowes, "US Cost-Utility Model of Lenacapavir Plus Optimized Background Regimen (OBR) vs Fostemsavir Plus OBR and Ibalizumab Plus OBR for People with HIV with Multidrug Resistance," *Journal of Managed Care & Specialty Pharmacy* 30, no. 9 (2024): 1001–1012, https://doi.org/10.18553/jmcp.2024.30.9.1001.

5. L. G. Bekker, M. Das, Q. Abdool Karim, et al., "Twice-Yearly Lenacapavir or Daily F/TAF for HIV Prevention in Cisgender Women," *New England Journal of Medicine* 391, no. 13 (2024): 1179–1192, https://doi.org/10.1056/NEJMoa2407001.

6. C. F. Kelley, M. Acevedo-Quiñones, A. L. Agwu, et al., "Twice-Yearly Lenacapavir for HIV Prevention in Men and Gender-Diverse Persons," *New England Journal of Medicine* 392, no. 13 (2025): 1261–1276, https://doi.org/10.1056/NEJMoa2411858.

7. N. Margot, V. Naik, A. Nekkalapudi, A. Boopathy, B. Falkard, and C. Callebaut, "Rapid HIV-1 Genotyping Assay for the Detection of Capsid Mutations," *Journal of Medical Virology* 95, no. 12 (2023): e29292, https://doi.org/10.1002/jmv.29292.

8. Q. Li, F. Yu, C. Song, et al., "HIV-1 Genotypic Resistance Testing Using Sanger and Next-Generation Sequencing in Adults With Low-Level Viremia in China," *Infection and Drug Resistance* 15 (2022): 6711–6722, https://doi.org/10.2147/IDR.S387215.

9. D. Armenia, M. M. Santoro, M. C. Bellocchi, et al., "Viral Resistance Burden and APOBEC Editing Correlate with Virological Response in Heavily Treatment-Experienced People Living with Multi-Drug Resistant HIV," *International Journal of Antimicrobial Agents* 59, no. 1 (2022): 106492, https://doi.org/10.1016/j.ijantimicag.2021.106492.

10. N. Stella-Ascariz, J. R. Arribas, R. Paredes, and J. Z. Li, "The Role of HIV-1 Drug-Resistant Minority Variants in Treatment Failure," *Journal of Infectious Diseases* 216, no. suppl_9 (2017): S847–S850, https://doi.org/10.1093/infdis/jix430.

11. A. Soria, C. Alteri, G. Scarlatti, et al., "Occupational HIV Infection in a Research Laboratory with Unknown Mode of Transmission: A Case Report," *Clinical Infectious Diseases* 64 (2016): 810–813, https://doi.org/10.1093/cid/ciw851.

12. M. C. Bellocchi, R. Scutari, L. Carioti, et al., "Frequency of Atypical Mutations in the Spike Glycoprotein in SARS-CoV-2 Circulating From July 2020 to July 2022 in Central Italy: A Refined Analysis by Next Generation Sequencing," *Viruses* 15, no. 8 (2023): 1711, https://doi.org/10.3390/v15081711.

13. C. Biba, L. Fiaschi, I. Varasi, et al., "A Comparison of Sanger Sequencing and Amplicon-Based Next Generation Sequencing Approaches for the Detection of HIV-1 Drug Resistance Mutations," *Viruses* 16, no. 9 (2024): 1465, https://doi.org/10.3390/v16091465.

14. T. Taylor, E. R. Lee, M. Nykoluk, et al., "A MiSeq-HyDRA Platform for Enhanced HIV Drug Resistance Genotyping and Surveillance," *Scientific Reports* 9, no. 1 (2019): 8970, https://doi.org/10.1038/s41598-019-45328-3.

15. D. Armenia, L. Carioti, V. Micheli, et al., "Comparison of Different HIV-1 Resistance Interpretation Tools for Next-Generation Sequencing in Italy," *Viruses* 16, no. 9 (2024): 1422, https://doi.org/10.3390/v16091422.

16. A. M. Bolger, M. Lohse, and B. Usadel, "Trimmomatic: A Flexible Trimmer for Illumina Sequence Data," *Bioinformatics* 30, no. 15 (2014): 2114–2120, https://doi.org/10.1093/bioinformatics/btu170.

17. B. M. P. Verbist, K. Thys, J. Reumers, et al., "VirVarSeq: A Low-Frequency Virus Variant Detection Pipeline for Illumina Sequencing Using Adaptive Base-Calling Accuracy Filtering," *Bioinformatics* 31, no. 1 (2015): 94–101, https://doi.org/10.1093/bioinformatics/btu587.

18. F. Madeira, N. Madhusoodanan, J. Lee, et al., "The EMBL-EBI Job Dispatcher Sequence Analysis Tools Framework in 2024," *Nucleic Acids Research* 52, no. W1 (2024): W521–W525, https://doi.org/10.1093/nar/gkae241.

19. WHO World Health Organization. WHO HIVResNet HIV Drug Resistance Laboratory Operational Framework. Second Edi. (WorldHealthOrganization, ed.). Licence: CC BY-NC-SA 3.0 IGO.; 2020.

20. A. D. Nka, Y. Bouba, G. Teto, et al., "Evaluation of HIV-1 Capsid Genetic Variability and Lenacapavir (GS-6207) Drug Resistance-Associated Mutations According to Viral Clades Among Drug-Naive Individuals," *Journal of Antimicrobial Chemotherapy* 78, no. 1 (2023): 272–275, https://doi.org/10.1093/jac/dkac388.

21. L. Fabeni, D. Armenia, I. Abbate, et al., "HIV-1 Transmitted Drug Resistance in Newly Diagnosed Individuals in Italy Over the Period 2015–21," *Journal of Antimicrobial Chemotherapy* 79, no. 9 (2024): 2152–2162, https://doi.org/10.1093/jac/dkae189.

Supporting Information

Additional supporting information can be found online in the Supporting Information section.
Supplementary Table 1.