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# The relevance of ultradeep sequencing for low HIV-1 viral loads and proviruses in the clinical setting

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## Abstract

Improving the therapeutic management of HIV-positive persons is a major public health issue and includes better detection of drug resistance mutations (DRMs). The aim of this study was (i) to explore DRMs in HIV-1-positive persons presenting a blood viral load (VL) < 1000 genomes copies (gc)/mL, including the analyze of cerebrospinal fluid (CSF) and HIV-DNA from peripheral blood mononuclear cells using ultradeep sequencing (UDS) and (ii), to evaluate how these DRMs could influence the clinical practices. For each patient ( $n = 12$ ), including five low-VL patients (i.e., <1000 gc/mL), HIV-1 UDS targeting the protease, reverse transcriptase and integrase genes was performed on plasma, proviral DNA, and CSF when available. Sequencing discordances or failures were mostly found in samples from low-VL patients. A 5% UDS cut-off allowed to increase the sensitivity to detect DRMs in different compartments, excepted in CSF. Patients with the highest viral quasispecies heterogeneity were naïve of treatment or presented a medical history suggesting low selection pressure or virological failures. When analyzing compartmentalization and following-up patients: low-frequency variants (LFVs) were responsible for 47% ( $n = 8$ ) and 76% ( $n = 13$ ) of changes in drug resistance interpretation, respectively. In such cases, we conclude that UDS is a robust technique, which still could be improved by increase the RNA and/or DNA extraction in low-VL samples to detect LFVs. Further studies are needed to define the impact of LFVs on anti-retroviral treatments. At last, when considering a DRM, the use of mutational load would probably be more suitable than frequencies.

## KEYWORDS

cerebrospinal fluid, drug resistance mutations, HIV-1, low-viral load, proviral DNA, ultradeep sequencing (UDS)

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## 1 | INTRODUCTION

A goal of the WHO is to end HIV infections and AIDS as a public health threat. This is one of the global health strategies for the period 2022–2030. One action targets HIV drug resistance and aims to develop laboratory capacity to monitor drug resistance mutations (DRMs).<sup>1</sup>

According to the guidelines, HIV-1 drug resistance testing can be performed at the time of diagnosis (before starting antiretroviral therapy [ARV]) and in the case of therapy failure, commonly performed on circulating viral RNA in plasma.

However, it can also be performed on HIV-DNA from peripheral blood mononuclear cells, in virologically suppressed patients before a change in treatment or in patients who present a low viral replication during treatment. A drug resistance test can also be performed to explore samples other than blood, especially cerebrospinal fluid (CSF) in which drug resistance can vary from plasma due to the uneven and poor penetration of ARV through the blood-brain barrier, associated with a possible local replication. Hence, the cause of treatment failure can be attributed to HIV-1 DRMs detected in CSF.<sup>2–4</sup>

Identification of DRMs, which may be associated with low viral load (VL) < 1000 genome copies (gc)/mL or needing the exploration of samples other than blood, requires the use of new tools but also to understand how to interpret these innovative results. Ultradeep sequencing (UDS) improves the performance of such analysis due to its greater analytical sensitivity for DRMs detection and its higher throughput. UDS also shows improved efficiency associated to an increasingly low cost.<sup>5–7</sup> However, the recent diagnostic HIV UDS kits are most often validated for high plasma VL (i.e., >1000 gc/mL), resulting in a lack of data for lower VL or for the archived DRMs within proviral DNA, which remain nevertheless key points in real life conditions.

In this study, we evaluated the performance of UDS to detect DRMs in low-VL samples as well as in different biological compartments (i.e., plasma, proviral DNA, and CSF). We also assessed how DRMs detection by UDS might influence clinical practice.

## 2 | MATERIALS AND METHODS

### 2.1 | Patients and samples

The study included 12 HIV-1-positive persons sampled between April and October 2022 at the University Hospital of Nancy (France). At the time of inclusion, all patients received an information letter explaining the objective of the research and the principle of non-opposition contained. Clinical and demographic data were collected from the Nadis<sup>®</sup> database (all the patients signed the ethics board-approved informed consent form of the HIV Nadis<sup>®</sup> electronic patient record database).

The samples have been sent to the virology laboratory as part of routine patient care, including HIV-1 RNA quantification and/or genotypic resistance testing on blood and/or CSF, and/or peripheral

blood mononuclear cells after centrifugation (2000 × g, 10 min) to collect proviral DNA (Table 1). Patients were selected based on their plasmatic VL (i.e., low-VL < 1000 gc/mL, *n* = 5 patients) or when a CSF or a second blood samples were available within a period of less than 2 weeks for the same patient (*n* = 7 patients, i.e., high-VL patients).

When the plasmatic VL was less than 100 gc/mL, only proviral DNA sequencing was performed. RNA and/or DNA genotyping were also performed on a follow-up blood sample if available and relevant, meaning collected during the care and with a detectable VL (*n* = 7 patients). At last, we recovered a second follow-up sample for one patient (patient #5) to carry out reproducibility analyses on low-VL plasma (144 gc/mL, Table 1).

### 2.2 | Quantification of HIV-1 viral load

HIV-1 RNA VL quantification was performed from plasma and CSF samples with the Aptima HIV-Quant Dx assay (Hologic) on a Panther<sup>™</sup> System device, with a detection limit of 30 gc/mL.

### 2.3 | HIV-1 sequencing

Genome extraction was performed using the NucliSENS EasyMag<sup>™</sup> device (BioMérieux) from 500 to 1000 μL of sample according to the manufacturer's recommendations. Nucleic acids were recovered in 40–100 μL of elution buffer, depending on VL.

Amplification of targeted regions (protease [PR], reverse transcriptase [RT], and integrase [IN]) was performed using DeepChek<sup>®</sup> PR/RT and DeepChek<sup>®</sup> INT nested-PCR assays (ABL S.A.), leading the production of 520 nt, 937 nt and 670 nt amplicons, respectively.

The amplicons were purified using Ampure Xp magnetic beads (Beckman Coulter) according to the manufacturer's recommendations.

UDS library construction was performed using DeepChek NGS Library Preparation Assay V2, and sequencing was performed using the iSeq. 100 platform (Illumina).

### 2.4 | UDS analyses

Sequence alignment, comparison, and data analysis were performed using DeepChek<sup>®</sup>-HIV1 v2.0, a downstream analysis software intended to be used for clinical interpretations. Reads obtained for each patient were aligned to the HIV-1 HXB2 reference sequence (access number K03455.1) and BLAST (basic local alignment search tool, NCBI) or COMET (Context-based Modeling for Expedious Typing, Luxembourg Institute of Health) was used on a per-region basis to determine patients' most likely subtype. Genotypic drug resistance interpretation was performed using the ANRS v32 algorithm (<https://www.hivfrenchresistance.org/>). DRMs were identified with an interpretation cut-off of 20% and 5%. The 20% cut-off was set because of the detection limit of the Sanger sequencing, the

**TABLE 1** Samples characteristics used for ultradeep sequencing (UDS) assays.

Patient	Samples	Compartments	Reproducibility assay (extraction + UDS)	RNA viral load (genomes copies/mL)	Sampling date
Patient #1	First sampling	Cerebrospinal fluid (CSF)	1	5064	May 09, 2022
		Plasma	1	2,156,033	May 06, 2022
		Proviral DNA	1	-	May 06, 2022
	Follow-up	Proviral DNA	1	-	July 05, 2022
Patient #2	First sampling	Plasma	1	465	April 05, 2022
		Proviral DNA	1	-	April 05, 2022
Patient #3	First sampling	CSF	1	56,298	June 03, 2022
		Plasma	1	536,976	June 07, 2022
		Proviral DNA	1	-	June 07, 2022
	Follow-up	Plasma	1	137	July 12, 2022
		Proviral DNA	3	-	July 12, 2022
Patient #4	First sampling	Plasma	1	278	May 25, 2022
		Proviral DNA	3	-	May 25, 2022
Patient #5	First sampling	Plasma	1	624	June 01, 2022
		Proviral DNA	1	-	June 01, 2022
	Follow-up	Plasma	1	471	August 11, 2022
		Proviral DNA	1	-	August 11, 2022
	2nd follow-up	Plasma	3	144	September 30, 2022
Patient #6	First sampling	CSF	1	30,162	October 12, 2022
		Plasma	1	575,715	October 13, 2022
		Proviral DNA	1	-	October 13, 2022
Patient #7	First sampling	CSF	1	1,003,874	May 25, 2022
		Plasma	1	4,486,953	May 12, 2022
		Proviral DNA	1	-	May 12, 2022
	Follow-up	Plasma	1	364	July 28, 2022
		Proviral DNA	1	-	July 28, 2022
Patient #8	First sampling	Plasma	1	837	July 21, 2022
		Proviral DNA	1	-	July 21, 2022
Patient #9	First sampling	Plasma	1	213	July 25, 2022
		Proviral DNA	1	-	July 25, 2022
	Follow-up	Proviral DNA	1	-	September, 07, 2022
Patient #10	First sampling	CSF	1	36,703	August 04, 2022
		Plasma	1	2,322,456	August 01, 2022
		Proviral DNA	1	-	August 01, 2022
	Follow-up	Plasma	1	105	September 09, 2022
		Proviral DNA	1	-	September 09, 2022
Patient #11	First sampling	CSF	1	686	August 23, 2022
		Plasma	3	349,575	August 11, 2022

(Continues)

TABLE 1 (Continued)

Patient	Samples	Compartments	Reproducibility assay (extraction + UDS)	RNA viral load (genomes copies/mL)	Sampling date
		Proviral DNA	1	-	August 11, 2022
	Follow-up	Proviral DNA	1	-	September 22, 2022
Patient #12	First sampling	CSF	1	47,921	August 27, 2022
		Plasma	1	9704	August 27, 2022
		Proviral DNA	1	-	August 27, 2022

current gold standard in HIV sequencing, while the 5% cut-off seems to be an optimal threshold to improve sensitivity and avoid false-positive DRMs when using UDS.<sup>8,9</sup> Low-frequency variants (LFVs) were defined as DRMs with frequencies between 5% and 20%.

Enrichment of mutations with APOBEC signatures was assessed using Hypermut 2.0 ([www.hiv.lanl.gov/content/sequence/HYPERMUT/hypermut.html](http://www.hiv.lanl.gov/content/sequence/HYPERMUT/hypermut.html)) on samples sequenced by UDS. Sequences with a *p*-value of 0.05 or less by the Fisher's exact test were considered significantly hypermutated and excluded from analyses.<sup>10</sup> One targeted region from only one sample was concerned.

At last, the mutational load (ML, corresponding to the number of copies of a mutation present in a given volume of fluid) was calculated by multiplying the viral load measured in the sample by the mutation frequency detected in the same sample.

## 2.5 | Quasispecies distance estimation

With the aim of approaching a dynamic of differential evolution of viral quasispecies depending on biological compartments, distances between viral quasispecies were obtained in plasma, CSF, and peripheral blood mononuclear cells (proviral DNA) with the Quasispecies Distance tool using Galaxy ([www.usegalaxy.fr/?tool\\_id=toolshed.g2.bx.psu.edu%2Frepos%2Fnm1%2Fquasitools%2Fdistance%2F0.7.0%20galaxy1](http://www.usegalaxy.fr/?tool_id=toolshed.g2.bx.psu.edu%2Frepos%2Fnm1%2Fquasitools%2Fdistance%2F0.7.0%20galaxy1)).<sup>11</sup> This tool allows reporting the distance between multiple read pileups from multiple quasispecies samples, aligned to the same genomic region. It determines the cosine relatedness between viral quasispecies, reporting angular cosine distance or cosine similarity as measures of relatedness. These measures should be understood as approximations for evolutionary distance.

## 2.6 | Statistical analyses

Data are presented as a median, lower quartile (Q1–25%) and upper quartile (Q3–75%). The Wilcoxon signed rank test was used for matched pairs. A statistically significant difference was defined as a *p*-value of < 0.05. The level of agreement during reproducibility

assays was calculated using the free-marginal multirater kappa<sup>12</sup> with a 5% and 10% cut-off. A result of above 0.900 was interpreted as almost perfect, 0.800–0.900 as strong agreement, 0.600–0.790 as moderate agreement, 0.400–0.590 as weak agreement and 0.200–0.390 as minimal agreement.<sup>13</sup>

## 3 | RESULTS

### 3.1 | Patient and sample characteristics

The patients were mainly male (10/12), with a median age of 50 years (Q1 45.75 – Q3 54.25). The main infecting virus was subtype B (9/12) (Table 2). At the time of sampling, the median plasma VL was  $4.65 \times 10^2$  gc/mL ( $2.78 \times 10^2$ – $6.24 \times 10^2$ ) for low-VL patients (*n* = 5) and  $5.76 \times 10^5$  gc/mL ( $4.43 \times 10^5$ – $2.24 \times 10^6$ ) for high-VL patients (*n* = 7) (Table 1); median CD4 counts were 245 cells/mm<sup>3</sup> (157–301) and 18 cells/mm<sup>3</sup> (13–44), respectively (Table 2). For high-VL patients, the median CSF VL was  $3.67 \times 10^4$  gc/mL ( $1.76 \times 10^4$ – $5.2 \times 10^4$ ) (Table 1). Patients with low-VL had been diagnosed for a median period of 5.5 years (0–9.5) while the median period was 4 days (0.5 days–16 years) for patients with high-VL (Table 2). For all included patients (high and low-VL), the median period between diagnosis and the start of treatment was 18 days (10.25–52.5) (Table 2).

Among the 12 patients, 43 samples were collected (i.e., 17 plasmas, 19 proviral DNA, and 7 CSF samples) and 51 UDS analyses were performed, including a reproducibility assay (Table 1). An optimal UDS was obtained for 86% of samples (*n* = 44), with a mean coverage of 129,931 reads (Table 3). Partial UDS (i.e., failure of one or two target genes) was obtained for 6 samples (i.e., two plasmas—278 and 364 gc/mL, respectively; one CSF sample—5064 gc/mL and three proviral DNA samples coming from low-VL patients), with a mean coverage of 144,310 reads. Failures occurred four times for the reverse transcriptase (RT), three times for the integrase (IN), and none for the protease (PR), defined by the absence of amplification or by the fact that the region did not reach the minimum coverage of 100 reads. A complete UDS failure was obtained for one sample (i.e., absence of amplification for a plasma—213 gc/mL, Table 3).

**TABLE 2** Patient characteristics.

	Patients	Age (years)	Sex	HIV-1 subtype	Time since HIV-1 diagnosis (years)	Time between diagnosis and start of treatment (days)	CD4 cell count (cells/mm <sup>3</sup> )	Drug therapy at first samples	Drug therapy at follow-up samples
Low viral load (<1000 gc/mL)	#2	52	F	B	29	1263	142	2 NRTIs + INI	No sampling
	#4	34	M	B	12	36	445	PI + NNRTI + INI	No sampling
	#5	45	M	B	0	19	245	2 NRTIs + INI	Identical
	#8	59	M	B	21	0	301	2 NNRTIs + PI	No sampling
	#9	46	M	B	4	11	157	2 NRTIs + INI	Identical
High viral load (>1000 gc/mL)	#1	47	F	CRF02	0	17	11	Naïve	2 NRTIs + INI
	#3	48	M	B	0	7	11	Naïve	2 NRTIs + INI
	#6	55	M	B	0	8	15	Naïve	No sampling
	#7	22	M	CRF02	7	52	18	2 NRTIs + PI	2 NRTIs + INI
	#10	54	M	A1	0	13	121	Naïve	2 NRTIs + INI
	#11	52	M	B	25	54	55	ARV interruption	2 NRTIs + INI
	#12	56	M	B	36	6879	33	ARV interruption	No sampling

Abbreviations: F, female; Gc, genomes copies; INI, integrase inhibitor; M, male; NNRTI, non-nucleoside reverse transcriptase inhibitor; NRTI, nucleoside reverse transcriptase inhibitor; PI, protease inhibitor.

### 3.2 | The use of UDS to study DRMs in different compartments

Plasma, CSF, and proviral DNA available for the seven high-VL patients and plasma and proviral DNA available for the four low-VL patients were used to study the distribution of DRMs. Low-VL follow-up samples (plasma and proviral DNA) from three patients with initially high-VL were also explored (Table 1).

For high-VL samples (Table 4A), DRMs were predominantly identified in the PR region (72%,  $n = 18$ ), followed by the RT (20%,  $n = 5$ ) and the IN (8%,  $n = 2$ ). For low-VL samples, the DRMs distribution was 41% ( $n = 13$ ), 41% ( $n = 13$ ), and 18% ( $n = 6$ ) for the PR, RT, and IN regions, respectively (Table 4B).

More mutations were detected in low-VL samples (21 DRMs including 5 LfVs in plasma; 27 DRMs including 8 LfVs in proviral DNA, Table 4B) compared to high-VL samples (18 DRMs including 4 LfVs in plasma; 21 DRMs including 4 LfVs in proviral DNA, Table 4A).

All in all, 10 DRMs were detected only in proviral DNA, including seven LfVs, and six were responsible for changes in drug resistance interpretation in different sequences: PR#7, PR#11, RT#10, RT#5, and IN#7 (Table 4). Furthermore, two DRMs were detected only in CSF, including one LfV which did not lead to changes in drug resistance interpretation (RT#11).

When DRMs were detected both in plasma and CSF, the frequency in CSF was mainly higher than that detected in plasma ( $n = 10/14$ ). However, when using the concept of ML, the quantity of genome copies affected by the DRMs was higher in plasma ( $n = 12/14$ ) (Table 4A).

The use of a 5% cut-off would supposed to increase the sensitivity of DRMs detection in the different compartments, as illustrate with the detection of the L10V (PR) in plasma (12.08%) and CSF (8.32%) at lower frequencies than in proviral DNA in patient #7, and the detection of the K20R (PR) in plasma (6.79%) and CSF (8.78%) in patient #12. Overall, this lower threshold resulted in the detection of 17 LfVs, among them 47% ( $n = 8$ ) were responsible for the change in drug resistance interpretation (Table 4 and Figure 1).

Interestingly, the ad-value of reducing the cut-off with the aim to increase the number of DRMs detected was significant in plasma and proviral DNA ( $p < 0.014$  Wilcoxon Signed-Rank Test) but not in CSF ( $p = 0.0832$ , Figure 2).

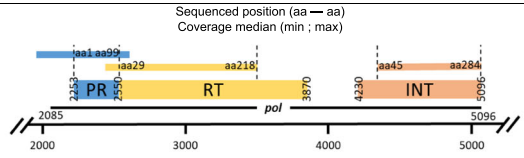
When compared the HIV quasiespecies distribution between biological compartments, a low heterogeneity was observed in most patients, except for patient #7 and especially for patient #1, characterized by the highest diversity (Figure 3).

### 3.3 | The use of UDS for patient follow-up

Follow-up samples were available from seven patients (i.e., five high-VL and two low-VL). All follow-up samples presented a low-VL  $< 471$  gc/mL, (Table 1).

After the introduction of ARV in naïve patients (i.e., #1, #3, and #10—2 nucleoside reverse transcriptase inhibitors [NRTI] + 1 integrase inhibitor [INI], Table 2), we observed the emergence of 2 LfVs conferring a resistance to NRTI and INI in patient #3 (Table 5A) after 32 days of treatment (Table 1). For patient #10, the follow-up sample collected after 26 days (Table 1) of ARV

TABLE 3 Sequencing coverage and position sequenced for each sample.



Patient	Sample	Compartment	Sequenced position (aa - aa)			Coverage median (min; max)				
			PR	RT	INT	PR	RT	INT		
#1	First sampling	CSF	1	99	1	3,660 (1,169; 5,205)	318	Failed	284	
		Plasma	1	99	1	3,537(2,380; 4,941)	318	45	2,768 (581; 4,678)	
		Proviral DNA	1	99	1	5,135 (0; 15,755)	327	37	14,781(0; 18,171)	
	Follow-up	Proviral DNA	1	99	1	<b>77 (0; 4,211)</b>	29	42	4,038 (34; 5,378)	
		CSF	1	99	1	4,186 (0; 21,178)	327	37	5,561 (0; 7,880)	
		Plasma	1	99	1	7,931 (2,965; 8,643)	327	45	4,750 (1198; 8,625)	
	#3	First sampling	Proviral DNA	1	99	1	8,447 (1; 13,219)	327	37	3,377 (1; 9,227)
			Plasma	1	99	1	4,866 (0; 17,992)	327	37	4,331 (0; 5,471)
			Proviral DNA	1	99	1	3,252 (0; 10,222)	327	37	2,357 (0; 3,615)
		Follow-up	Proviral DNA (extraction 1)	1	99	1	11,633 (0; 20,915)	326	37	7,385 (4; 8,663)
			Proviral DNA (extraction 2)	1	99	1	12,989 (0; 27,372)	326	37	10,506 (5; 12,851)
			Proviral DNA (extraction 3)	1	99	1	12,989 (0; 27,372)	326	37	10,506 (5; 12,851)
#6		First sampling	CSF	1	99	1	1,922 (0; 7,655)	326	37	2,261 (0; 4,194)
			Plasma	1	99	1	8,626 (0; 27,579)	327	37	9,084 (1; 12,659)
			Proviral DNA	1	99	1	1,518 (0; 19,437)	326	37	11,115 (66; 17,989)
		Follow-up	CSF	1	99	1	2,857 (4; 11,156)	326	38	1,126 (52; 2,216)
			Plasma	1	99	1	2,682 (0; 4,489)	326	46	1,630 (1; 2,029)
			Proviral DNA	1	99	1	3,750 (0; 25,187)	327	37	3,798 (212; 5,909)
	#7	First sampling	Plasma	1	99	1	<b>25 (0; 31,891)</b>	29	37	14,245 (18; 20,425)
			Proviral DNA	1	99	1	<b>18 (0; 30,268)</b>	29	37	8,105 (539; 10,868)
			CSF	1	99	1	2,859 (0; 7,521)	326	37	6,305 (2; 8,030)
		Follow-up	Plasma	1	99	1	1,780 (0; 5,158)	326	37	5,023 (3; 6,208)
			Proviral DNA	1	99	1	15,427 (0; 23,474)	326	37	16,138 (34; 19,909)
			Plasma	1	99	1	10,084 (0; 26,802)	327	37	4,265 (5; 5,604)
#10		First sampling	CSF	1	99	1	5,527 (0; 11,031)	326	37	4,674 (4; 5,601)
			Plasma	1	99	1	4,529 (0; 5,592)	326	37	2,299 (380; 5,174)
			Proviral DNA	1	99	1	765 (0; 4,292)	326	37	4,284 (1; 5,196)
		Follow-up	Plasma (extraction 1)	1	99	1	9,576 (0; 24,875)	326	37	10,182 (4; 12,106)
			Plasma (extraction 2)	1	99	1	8,813 (0; 27,926)	326	37	7,449 (2; 9,139)
			Plasma (extraction 3)	1	99	1	1,172 (0; 3,050)	326	37	3,665 (0; 4,402)
	#11	First sampling	Proviral DNA	1	99	1	7,787 (1; 12,124)	326	37	5,805 (220; 6,891)
			CSF	1	99	1	1,507 (0; 6,006)	326	37	8,261 (4; 10,758)
			Plasma	1	99	1	3,752 (0; 8,531)	327	37	6,866 (0; 16,084)
		Follow-up	Proviral DNA	1	99	1	3,366 (0; 5,558)	327	37	6,462 (0; 9,080)
			Plasma	1	99	1	1,124 (894; 5,214)	318	45	6,175 (1,573; 7,818)
			Proviral DNA	1	99	1	5,881 (3; 16,415)	327	37	5,104 (11; 7,867)
#4		First sampling	Plasma	1	99	1	Failed	Failed	Failed	Failed
			Proviral DNA (extraction 1)	1	99	1	5,007 (1,790; 6,515)	318	55	6,802 (0; 8,633)
			Proviral DNA (extraction 2)	1	99	1	17,158 (0; 29,146)	326	40	8,739 (79; 11,375)
		Follow-up	Proviral DNA (extraction 3)	1	99	1	16,154 (2; 34,287)	326	37	10,271 (2; 12,800)
			Plasma	1	99	1	4,662 (0; 8,835)	327	37	6,707 (4; 8,223)
			Proviral DNA	1	99	1	4,065 (0; 9,397)	327	37	2,886 (0; 3,836)
	#5	First sampling	Plasma	1	99	1	4,457 (0; 27,058)	327	37	7,267 (5; 9,827)
			Proviral DNA	1	99	1	7,954 (354; 20,527)	327	37	10,007 (18; 17,943)
			Plasma (extraction 1)	1	99	1	2,128 (1,606; 8,439)	318	55	1,924 (0; 2,503)
		2nd follow-up	Plasma (extraction 2)	1	99	1	3,197 (0; 15,154)	326	37	9,872 (7; 13,204)
			Plasma (extraction 3)	1	99	1	3,704 (0; 16,738)	326	37	7,320 (2; 10,062)
			Proviral DNA	1	99	1	1,970 (5; 3,826)	326	37	2,918 (74; 3,714)
#8		First sampling	Plasma	1	99	1	3,681 (0; 12,867)	327	Failed	Failed
			Proviral DNA	1	99	1	Failed	Failed	Failed	Failed
			Plasma	1	99	1	9,885 (6,882; 10,691)	318	55	721 (95; 870)
		Follow-up	Proviral DNA	1	99	1	2,268 (1,419; 6,507)	318	45	6,973 (1,039; 7,972)
			Plasma	1	99	1	9,099 (5,143; 10,755)	318	45	6,973 (1,039; 7,972)
			Proviral DNA	1	99	1	11,625 (9,859; 12,752)	318	45	6,973 (1,039; 7,972)

Note: Results in bold indicates a sequencing failure.

Abbreviations: INT, integrase; PR, protease; RT, reverse transcriptase.

**TABLE 4** Drug resistance mutations detected in patient compartments using ultradeep sequencing with a 20% or 5% cut-off.

<b>(A) High-viral load patients</b>					
HIV-1 region	Patient	Mutations			Change in drug resistance interpretation
		Plasma	Proviral DNA	Cerebrospinal fluid (CSF)	
PR	#1	G16E (98.49%– $2.15 \times 10^5$ gc/mL), <u>L10V (96.08%) *</u>	G16E (97.91%), <u>L10V (91.66%) *</u>	G16E (98.71%– $4.94 \times 10^3$ gc/mL)	S → S/I
	#3	D60E (51.93%– $2.79 \times 10^5$ gc/mL), L63P (64.89%– $3.48 \times 10^5$ gc/mL), <u>A71V (35.31%), A71T (8.72%) *</u>	D60E (54.63%), L63P (69.85%), <u>A71V (30.08%) *, A71T (10.52%) *</u>	D60E (93.99%– $5.29 \times 10^4$ gc/mL), L63P (98.72%– $5.63 \times 10^4$ gc/mL)	S → S/I
	#6	A71T (98.84%– $5.69 \times 10^5$ gc/mL)	A71T (97%)	A71T (98.04%– $2.96 \times 10^4$ gc/mL)	No change
	#7	<u>L10V (12.08%–<math>5.42 \times 10^5</math> gc/mL)</u>	L10V (77.98%), <u>G16E (73.93%) *</u>	<u>L10V (8.32%–<math>8.35 \times 10^4</math> gc/mL)</u>	S → S/I
	#10	G16E (88.56%– $2.06 \times 10^6$ gc/mL), K20R (95.21%– $2.21 \times 10^6$ gc/mL)	G16E (71.16%), K20R (96.41%)	G16E (85.63%– $3.14 \times 10^4$ gc/mL), K20R (95.38%– $3.5 \times 10^4$ gc/mL)	No change
	#11	G16E (94.22%– $3.29 \times 10^5$ gc/mL), L33F (93.2%– $3.26 \times 10^5$ gc/mL), L63P (94.1%– $3.29 \times 10^5$ gc/mL)	G16E (97.5%), L33F (93.09%), L63P (97.32%), <u>M46L (7.66%) *</u>	G16E (96.39%– $6.61 \times 10^2$ gc/mL), L33F (90.05%– $6.18 \times 10^2$ gc/mL), L63P (97.44%– $6.69 \times 10^2$ gc/mL)	S/I → S/I/R
	#12	L63P (96.9%– $9.4 \times 10^3$ gc/mL), <u>K20R (6.79%–<math>6.58 \times 10^2</math> gc/mL)</u>	L63P (97.81%)	L63P (97.42%– $4.67 \times 10^4$ ), <u>I85V (23.91%), K20R (18.32% - <math>8.78 \times 10^3</math> gc/mL)</u>	No change
RT	#1	None	<u>V179I (55.23%, NNRTI)</u>	None	No change
	#10	E138A (98.15%– $2.28 \times 10^6$ gc/mL, NNRTI)	E138A (99.14%, NNRTI), <u>M184I (5.23%, NRTI) *, M230I (5.25%, NNRTI)</u>	E138A (99.19%– $3.64 \times 10^4$ gc/mL, NNRTI)	NRTI: S → S/I/R NNRTI: no change
	#11	None	None	<u>K103R (5.24%, NNRTI)</u>	No change
IN	##10	L74I (98.77%– $2.29 \times 10^6$ gc/mL), <u>Q148K (5.12%) *</u>	L74I (99.24%)	L74I (99.05%– $3.64 \times 10^4$ gc/mL)	S → I/R
<b>(B) Low-viral load patients</b>					
HIV-1 region	Patient	Mutations			Change in drug resistance interpretation
		Plasma	Proviral DNA		
PR	#2	L63P (99.34%)	<u>L10I (20.05%), L63P (98.68%)</u>		No change
	#4	L63P (99.38%)	L63P (99.61%), <u>M46I (8.06%)</u>		No change
	#8	L63P (99.2%)	L63P (98.79%)		No change
	#3 (follow-up)	D60E (57.41%), L63P (72%), A71V (25.63%), <u>A71T (8.1%)</u>	D60E (59.84%), L63P (64.68%), A71V (32.31%), <u>K65E (9.91%)</u>		No change
	#7 (follow-up)	None	<u>L10V (12.6%)</u>		No change
	#10 (follow-up)	G16E (96.76%), K20R (98.46%)	G16E (64.86%), K20R (98.38%)		No change
	RT	#4	Failed	D67N (51.05%, NRTI), K70E (51.39%, NRTI), <u>M184V (19.05%, NRTI)</u>	
#5		V179I (98.32%, NNRTI)	V179I (81.2%, NNRTI), <u>M230I (5.72%, NNRTI) *</u>		S → S/R
#8		L74V (25.4%, NRTI), L100I (26.2%, NNRTI), K103N (25.79%, NNRTI), V179I (22.8%, NNRTI),	L74V (38.45%, NRTI), L100I (36.91%, NNRTI), K103N (36.65%, NNRTI), V179I (30.4%, NNRTI)		No change

(Continues)



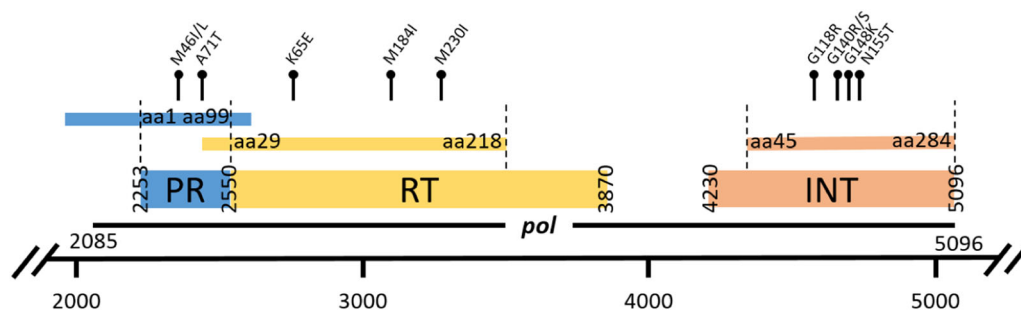
TABLE 4 (Continued)

(B) Low-viral load patients				
HIV-1 region	Patient	Mutations		Change in drug resistance interpretation
		Plasma	Proviral DNA	
		<b><u>E44D (11.26%, NRTI)</u></b>		
	#10 (follow-up)	E138A (99.02%, NNRTI)	E138A (99%, NNRTI), <b><u>M230I (23.35%, NNRTI)</u></b> , <b><u>M184I (19.22%, NRTI)*</u></b>	NRTI: S → S/I/R NNRTI: no change
IN	#5	<b><u>Q148K (5.36%)*</u></b> , <b><u>N155T (9.98%)*</u></b>	None	S/R → S
	#3 (follow-up)	Q148K (5.71%)	Q148K (6.28%)	No change
	#7 (follow-up)	L74I (98.54%)	<b><u>G140R (5.71%)*</u></b>	S → S/R
	#10 (follow-up)	L74I (98.7%)	L74I (99.25%)	No change

Note: Differences are underlined (i.e., mutation not detected in all compartments). Mutations detected at a 5% cut-off are in **bold**; mutational loads are in *italics* (genomes copies (gc)/mL). None, no mutation detected.

Abbreviations: IN, integrase; NRTI, nucleoside reverse transcriptase inhibitor; NNRTI, non-nucleoside reverse transcriptase inhibitor; PR, protease; RT, reverse transcriptase.

\*Indicates that differences in mutation causes change in the drug resistance interpretation (S: susceptible; I: possible resistance; R: resistance).



**FIGURE 1** Low-frequency variants (LFVs) responsible for changes in drug resistance interpretation. INT, integrase; PR, protease; RT, reverse transcriptase.

brought to light the archiving of the M184I and M230I in the proviral DNA (19.22% and 23.35%, respectively), conferring resistance to NRTI and non-nucleoside reverse transcriptase inhibitor (NNRTI), respectively. The Q148K mutation responsible for resistance to all INI was found only in the first plasma sample (5.12%), unlike the L74I (Table 5A). At last, for patient #1, we observed the emergence of the archived mutation M46I in the follow-up proviral DNA (5.03%, Table 5A), 47 days after introduction of ARV (Table 1).

When an ARV switch was made for patient #7 with the introduction of bictegravir (Table 2), the L10V mutation was no longer detected 43 days after the change of treatment (Table 1), but one LFV conferring resistance to INI was detected in the proviral DNA (G140R, 5.71%, Table 5A).

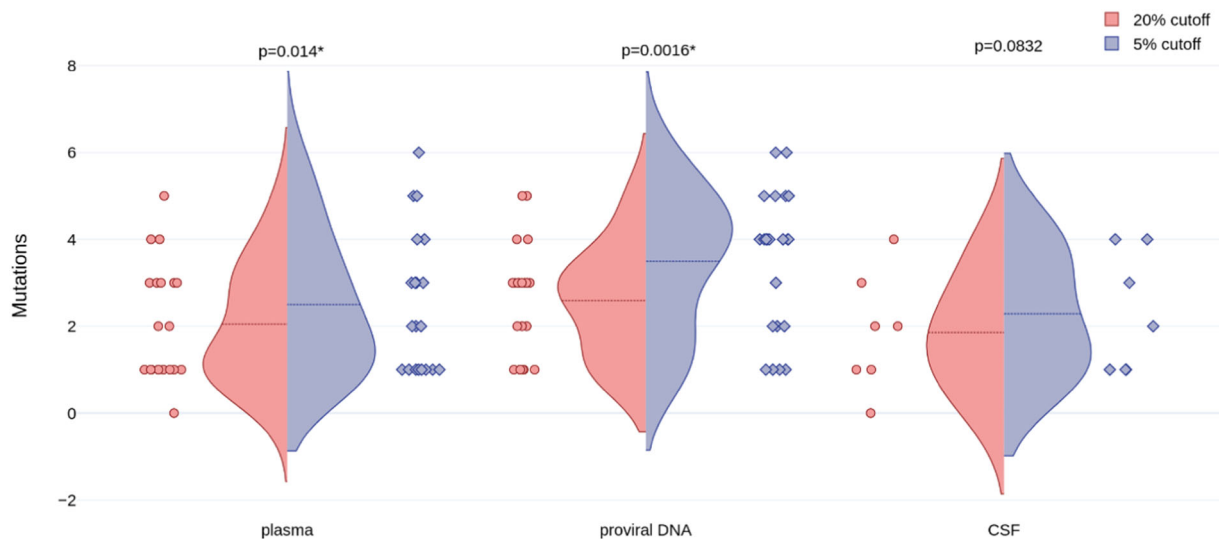
Patient #11 started a new treatment (2 NRTI + INI, Table 2) due to noncompliance, resulting in archiving the G140S (6.76%,

Table 5A) associated with resistance to INI, 30 days after the new treatment (Table 1).

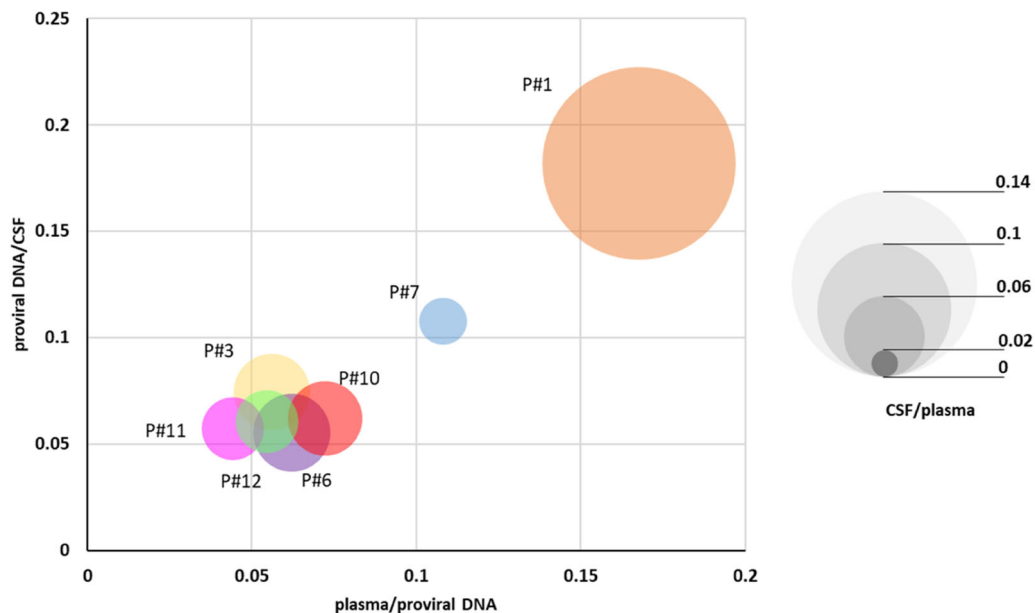
In the absence of treatment modification for patients #5 and #9 during the follow-up (2 NRTI + INI, Table 2), we observed for patient #5 the loss of the archived M230I initially detected at 5.72% and the loss of the Q148K (5.36%) and N155T (9.98%) initially present in the plasma. For patient #9, we observed the emergence of the archived mutations M230I (5%), G118R (9.53%), and Q148K (6.36%) which are responsible for resistance to NNRTI and INI (Table 5B).

In summary, 76% ( $n = 13$ ) of the LFVs detected in all samples were responsible for changes in drug resistance interpretation (Table 5 and Figure 1).

Among the 13 DRMs detected throughout the follow-up, 9 were still present in the second sampling with a similar frequency, despite the lower VL. In this case, the calculation of the ML allowed ensuring the relative decline of each DRMs, including LFVs (Table 5).



**FIGURE 2** Number of drug resistance mutations detected in plasma, proviral DNA and cerebrospinal fluid (CSF) using ultradeep sequencing. Dotted lines represent the average number of mutations with a 20% (red) and 5% cut-off (blue).  $p$ -values were calculated using the Wilcoxon Signed-Rank Test.  $p < 0.05$  are considered significant (\*).



**FIGURE 3** Evolutionary distance between the viral quaspecies isolated from the different biological compartments (i.e. plasma, proviral DNA, and cerebrospinal fluid [CSF]) in high-VL patients. The analysis was performed with the Quasispecies Distance tool using Galaxy ([www.usegalaxy.fr/?tool\\_id=toolshed.g2.bx.psu.edu%2Frepos%2Fnm%2Fquasitools%2Fdistance%2F0.7.0%20galaxy1](http://www.usegalaxy.fr/?tool_id=toolshed.g2.bx.psu.edu%2Frepos%2Fnm%2Fquasitools%2Fdistance%2F0.7.0%20galaxy1)).

### 3.4 | Reproducibility

A reproducibility assay was performed using two proviral DNA samples from low plasma-VL (patient #3, #4) and two plasmas, from low-VL-patient #5 follow-up and high-VL patient #11 (Table 1). As shown in Table 6, discordance in proviral DNA only concerned the LRVs, and the use of a 10% cut-off eliminates the qualitative heterogeneity observed (free-marginal kappa = 1.00). Concerning plasmas, no DRM was detected under a 20% cut-off, but one discordance remained detected in the RT for the low-VL patient #5 (144 gc/mL),

leading to a minimal agreement (free-marginal kappa = 0.33). Results were optimal concerning high-VL (free-marginal kappa = 1.00).

### 3.5 | The ad-value of UDS in clinical settings

The results of the genotypic resistance testing were correlated with the patient's medical history. Practically, UDS allowed to detect the L74V mutation in the RT gene in patient #8, responsible for abacavir resistance (Table 4B). This patient had a history of virological failure under this drug.

**TABLE 5** Drug resistance mutations evolution in follow-up samples from patients with high and low viral loads, using a 20% and 5% ultradeep sequencing cut-off.

<b>(A) High-viral load patients</b>						
HIV-1 region	Patient number	1st plasma	Follow-up plasma	1st proviral DNA	Follow-up proviral DNA	Change in drug resistance interpretation
PR	#1	L10V (96.08%), G16E (98.49%)	NA	L10V (91.66%), G16E (97.91%)	L10V (96.47%), G16E (93.53%), <b>M46I (5.03%)*</b>	S/I → S/R
	#3	D60E (51.93%; $2.79 \times 10^5$ gc/mL), L63P (64.89%; $3.48 \times 10^5$ gc/mL), A71V (35.31%; $1.90 \times 10^5$ gc/mL), <b>A71T (8.72%; <math>4.68 \times 10^4</math> gc/mL)</b>	D60E (57.41%; $79$ gc/mL), L63P (72%; $99$ gc/mL), A71V (25.63%; $35$ gc/mL), <b>A71T (8.1%; <math>12</math> gc/mL)</b>	D60E (54.63%), L63P (69.85%), A71V (30.08%), <b>A71T (10.52%)</b>	D60E (59.84%), L63P (64.68%), A71V (32.31%)	No change
	#7	<b>L10V (12.08%)</b>	None	L10V (77.98%), <b>G16E (73.93%)*</b>	<b>L10V (12.6%)</b>	S/I → S
	#10	G16E (88.56%; $2.06 \times 10^6$ gc/mL), K20R (95.21%; $2.21 \times 10^6$ gc/mL)	G16E (96.76%; $10^2$ gc/mL), K20R (98.46%; $10^3$ gc/mL)	G16E (71.16%), K20R (96.41%)	G16E (64.86%), K20R (98.38%)	No change
	#11	G16E (94.22%), L33F (93.2%), L63P (94.1%)	NA	G16E (97.05%), L33F (93.09%), L63P (97.32%), <b>M46L (7.66%)*</b>	G16E (87.85%), L33F (87.29%), L63P (98.75%)	S/I/R → S/I (atazanavir/r R → I)
RT	#1	None	NA	V179I (55.23%, NNRTI)	Failure	No change
	#3	None	None	None	<b>K65E (9.91%, NRTI)*</b>	NRTI: S → S/R NNRTI: no change
	#10	E138A (98.15%; $2.28 \times 10^6$ gc/mL, NNRTI)	E138A (99.02%; $10^4$ gc/mL, NNRTI)	E138A (99.14%, NNRTI), <b>M230I (5.25%, NNRTI), M184I (5.23%, NRTI)</b>	E138A (99%, NNRTI), M230I (23.35%, NNRTI), <b>M184I (19.22%, NRTI)</b>	No change
IN	#3	None	<b>Q148K (5.71%)*</b>	None	<b>Q148K (6.28%)*</b>	S → S/R
	#7	None	<b>L74I (98.54%)</b>	None	<b>G140R (5.71%)*</b>	S → S/R
	#10	L74I (98.77%; $2.29 \times 10^6$ gc/mL), <b>Q148K (5.12%)*</b>	L74I (98.7%; $10^2$ gc/mL)	L74I (99.24%)	L74I (99.25%)	I/R → S
	#11	None	NA	None	<b>G140S (6.76%)*</b>	S → S/R
<b>(B) Low-viral load patients</b>						
HIV-1 region	Patient number	Plasma	Follow-up plasma	Proviral DNA	Follow-up proviral DNA	Change in drug resistance interpretation
PR	#9	Failure	NA	L63P (98.72%)	L63P (96.73%)	No change
RT	#5	V179I (98.32%; $614$ gc/mL, NNRTI)	V179I (98.92%; $465$ gc/mL, NNRTI)	V179I (81.2%, NNRTI), <b>M230I (5.72%, NNRTI)*</b>	V179I (98.81%, NNRTI)	NNRTI:S/R → S NRTI: no change
	#9	Failure	NA	None	<b>M230I (5%, NNRTI)*</b>	NNRTI:S → S/R NRTI: no change
IN	#5	<b>Q148K (5.36%)*</b> <b>N155T (9.98%)*</b>	None	None	None	S/R → S
	#9	Failure	NA	<b>T97A (90.85%)</b>	<b>G118R (9.53%)*</b> <b>Q148K (6.36%)*</b>	S/R → R

Note: Differences are underlined (i.e., drug resistance mutations not detected in both first and follow-up samples). Mutations detected at a 5% cut-off are in bold; mutational loads are in italics (genomes copies (gc)/mL). None, no mutation detected; NA, no sample available.

Abbreviations: IN, integrase; NRTI, nucleoside reverse transcriptase inhibitor; NNRTI, non-nucleoside reverse transcriptase inhibitor; PR, protease; RT, reverse transcriptase.

\*Indicates that differences in mutation causes change in the drug resistance interpretation (S: susceptible; I: possible resistance; R: resistance).

TABLE 6 Reproducibility of HIV drug resistance mutation detection using ultradeep sequencing (UDS) with a 10% and 5% cut-off, on plasma and proviral DNA.

Reproducibility assay	Proviral DNA #3			Proviral DNA #4			Low-VL #5			high-VL #11		
	1	2	3	1	2	3	1	2	3	1	2	3
PR	D60E (59.84%), L63P (64.68%), A71V (32.31%)	D60E (57.98%), L63P (67.71%), A71V (33.28%)	D60E (51.17%), L63P (62.54%), A71V (35.34%)	L63P (99.61%), <b>M46I (8.06%)</b>	L63P (98.66%)	L63P (98.9%)	None	None	None	G16E (94.22%), L33F (93.2%), L63P (94.1%)	G16E (97.54%), L33F (96.28%), L63P (98.52%)	G16E (96.69%), L33F (95.62%), L63P (98.88%)
RT	<b>K65E</b> ( <b>9.91%</b> , <b>NRTI</b> )*	<b>M230I</b> ( <b>9.17%</b> , <b>NNRTI</b> )*	<b>M230I</b> ( <b>7.69%</b> , <b>NNRTI</b> )*	D67N (51.05%, NRTI), K70E (51.39%, NRTI), <b>M184V</b> ( <b>19.05%</b> , NRTI)	D67N (98.92%, NRTI), K70E (97.84%, NRTI), <b>M184V</b> ( <b>12.39%</b> , NRTI)	D67N (27.08%, NRTI), K70E (76.69%, NRTI), M184V (21.13%, NRTI)	T69N (24.81%, NRTI), V179I (97.03%, NNRTI)	V179I (98.75%, NNRTI)	V179I (98.76%, NNRTI)	None	None	None
IN	<b>Q148K</b> ( <b>6.28%</b> )*	None	None	None	None	None	None	None	None	None	None	None
Percent overall agreement (%)	66.67% (5% cut-off) 100.00% (10% cut-off)	66.67% (5% cut-off) 100.00% (10% cut-off)	86.67% (5% cut-off) 100.00% (10% cut-off)	66.67% (5% cut-off) 100.00% (10% cut-off)	66.67% (5% cut-off) 100.00% (10% cut-off)	66.67% (5% cut-off) 100.00% (10% cut-off)	66.67% (5% cut-off) 66.67% (10% cut-off)	66.67% (5% cut-off) 66.67% (10% cut-off)	66.67% (5% cut-off) 66.67% (10% cut-off)	100.00% (5% cut-off) 100.00% (10% cut-off)	100.00% (5% cut-off) 100.00% (10% cut-off)	100.00% (5% cut-off) 100.00% (10% cut-off)
Free-marginal kappa [95% confidence interval]	0.33 [-0.25; 0.92] (5% cut-off) 1.00 [1.00, 1.00] (10% cut-off)	0.33 [-0.25; 0.92] (5% cut-off) 1.00 [1.00, 1.00] (10% cut-off)	0.73 [0.21, 1.00] (5% cut-off) 1.00 [1.00, 1.00] (10% cut-off)	0.33 [-0.97, 1.00] (5% cut-off) 0.33 [-0.97, 1.00] (10% cut-off)	0.33 [-0.97, 1.00] (5% cut-off) 0.33 [-0.97, 1.00] (10% cut-off)	0.33 [-0.97, 1.00] (5% cut-off) 0.33 [-0.97, 1.00] (10% cut-off)	0.33 [-0.97, 1.00] (5% cut-off) 0.33 [-0.97, 1.00] (10% cut-off)	0.33 [-0.97, 1.00] (5% cut-off) 0.33 [-0.97, 1.00] (10% cut-off)	0.33 [-0.97, 1.00] (5% cut-off) 0.33 [-0.97, 1.00] (10% cut-off)	1.00 [1.00, 1.00] (5% cut-off) 1.00 [1.00, 1.00] (10% cut-off)	1.00 [1.00, 1.00] (5% cut-off) 1.00 [1.00, 1.00] (10% cut-off)	1.00 [1.00, 1.00] (5% cut-off) 1.00 [1.00, 1.00] (10% cut-off)

Note: Discordances are underlined (i.e., mutations not detected in all extracts). Mutations detected at a 5% cut-off are in **bold**. None, no mutation detected.

Abbreviations: IN, integrase; NRTI, nucleoside reverse transcriptase inhibitor; NNRTI, non-nucleoside reverse transcriptase inhibitor; PR, protease; RT, reverse transcriptase.

\*Indicates that discordances in mutation causes change the drug resistance interpretation.

In patient #11, who had a history of virological failure with protease inhibitor (PI), DRMs were found in the PR region, including a LFV responsible for changes in the interpretation of drug resistance (M46L, 7.66%, Table 4A). In patient #4 presenting a history of virological failure with NRTI, UDS detected an archived M184V mutation responsible for several NRTI resistance (Table 4B). Finally, during the patient #7 follow-up, UDS detected a LFV conferring resistance to INI after the introduction of bicitgravir, associated with the virological failure (G140R, 5.71%, Table 4B).

## 4 | DISCUSSION

By improving the therapeutic management of HIV-positive persons, UDS represents the future of HIV genotyping. However, the routine use of this technique still shows some limitations, including the clinical significance of LFVs. If their detection is largely indicated in naïve patient receiving ARV with a weak genetic barrier (i.e., NNRTI), their relevance remains discussed for patients already treated with ARV presenting a high genetic barrier. However, such patients represent the most difficult to treat and require hence sensitive analyses (such as UDS) in the case of virological failure.<sup>14</sup> Furthermore, the description of pre-existing LFVs in naïve patients responsible for virological failure to first-line NNRTI-based ARV is also an argument in favor of the routine use of UDS.<sup>15,16</sup> Nevertheless, how LFVs should be taken into account in patients with low-VL and potentially in the case of virological failure remains unclear, as illustrated by the example that a LFV present at a rate of 1% would require HIV-1 RNA levels of at least 1000 gc/mL to be detected effectively.<sup>14</sup>

In this study, we explored various situations in which UDS could provide and ad-value, especially concerning the analysis of low-VL samples (<1000 gc/mL) and the exploration of other compartments than blood. This work has the limitation of being carried out on a small number of patients and other studies are needed to improve the position of UDS to monitor HIV-positive persons. However, some observations can be drawn.

First, we observed that a threshold could not precisely be set for UDS success, since samples with VL as low as 105 gc/mL have been sequenced, and an optimal sequencing was obtained for 86% of samples ( $n = 44$ ) for the sequencing of HIV PR, RT and IN, including 12 low VL samples (75% of success). According to the manufacturer recommendations, the threshold allowing the best analytical sensitivity is 1000 gc/mL, and a procedure is available for lowering to 200–300 gc/mL-VL. Overall, previous data reported a success rate of over 84% on viral loads between 2 and 3  $\log_{10}$  gc/mL using the same approach.<sup>17</sup> By focusing on the technical process, HIV sequencing requires a prior RNA extraction step, and a sufficient quantity of viral RNA is necessary to ensure an accurate representation of the DRMs. However, selection of RNA molecules following a Poisson distribution with stochastic sampling effects inversely proportional to the number of RNA molecules, it is therefore easy to conclude that low-VL sequencing may results to underestimate the presence of some DRMs, especially LFVs.<sup>18</sup> This may explain discordances observed in low-VL samples and in this context, it would be relevant to carry out several viral RNA extractions to increase the fidelity

of LFVs distribution in patients with low-VL, by increasing the sequencing runs.

Once the question of sensitivity addressed, the next step concerns the relevance of detecting such mutations. For this second aspect, the use of the ML seems to be relevant to explore the emergence of DRMs during patient follow-up, but also to compare their presence in different compartments within a patient. This raises the question of introducing the VL parameter into the interpretation of DRMs frequencies and their impact on the virological response.<sup>19</sup> Previous studies have already shown a relationship between virological failure associated to NNRTIs and ML<sup>15,19</sup> and currently, a clinical threshold of 2000 gc/mL is classically accepted for the presence of the K103N mutation and the risk of virological failure associated to first generation NNRTI.<sup>20</sup>

Within biological compartments, the differences of DRMs distribution between CSF and plasma were expected and may be the result of local replication.<sup>4</sup> In this context, previous studies have demonstrated a good correlation between the results obtained by the Sanger method and by UDS in patients presenting symptoms consistent with HIV encephalopathy.<sup>21</sup> However, the clinical significance of LFVs in CSF remains unknown, and according to our results (Figure 2) it would seems that the use of a 5% cut-off is not necessary and as presented herein, the use of ML should also be considered.<sup>22</sup>

Discordances between DRMs present in proviral DNA and plasma were also expected and mostly result from the variable kinetics of mutations in the reservoir (e.g., presence of mutations not always archived, quantitatively and qualitatively nonhomogeneous distribution of the variants in the cells and APOBEC context mutations).<sup>23,24</sup> In the reproducibility analyses performed for patient #4, a lower threshold (i.e., 10%) allowed to detect the M184V mutation in all three DNA extractions, despite their differences in frequencies. Figure 2 shows the importance of using a cut-off lower than 20% for proviral DNA analysis, improving the detection of DRMs in patients who have already been treated, as previously reported.<sup>25</sup> However, a single sequencing seems not always sufficient for this type of sample, and here again, multiple DNA extractions would increase the sensitivity for a more complete view of the archived DRMs.<sup>23</sup>

In this work, patients presenting the highest quasispecies heterogeneity within each biological compartment were also screened for their medical history of HIV infection, looking for contexts compatible with a low selection pressure before ARV or for previous virological failure: Patient #7 had been infected for several years with multiple episodes of therapeutic failure. On the opposite, patient #1 which also presented a high quasispecies heterogeneity between the different compartments was supported for a discovery of HIV infection, not yet received ARV, and did not present any major DRMs.

At last, our results highlight the benefit of UDS in clinical settings. First, 47% of LFVs present in compartments other than plasma were responsible for changes in drug resistance interpretation and might therefore be responsible for switching treatment.

Second, we observed the effectiveness of routine UDS during follow-up, especially after the introduction of ARV or switch. In these situations, 76% of LFVs detected were responsible for a change in drug resistance interpretation. The change in drug resistance interpretation by reducing the DRMs detection cut-off <20% has already been

demonstrated in previous studies, but the clinical impact of these changes remains to be proven.<sup>17</sup> Moreover, UDS allowed us to detect mutations of interest in four patients with a history of virological failure, including LFVs.

Thanks to a better sensitivity, UDS allows earlier detection of potential LFVs responsible for resistance in patients on ARV, as well as in naïve patients. For the latter, a recent meta-analysis including 15,242 patients concluded to an optimal threshold close to 2% or 5% to detect DRMs.<sup>26</sup> This threshold was also approved here to be a good compromise during the exploration of low-VL samples, CSF or peripheral blood mononuclear cells, improving the sensitivity while limiting false-positive resistance and ensuring satisfactory reproducibility.

## 5 | CONCLUSION

In conclusion, UDS is a robust technique for monitoring HIV-positive persons. The use of a 5% cut-off allows clinicians to access to LFVs and thus to acquire more information to potentially adapt the ARV. In the current state of knowledge, only detection of LFVs affecting NNRTIs in naïve patients is routinely admitted and the use of ML will probably be more suitable than DRMs frequencies. The improved analytical sensitivity of the UDS should also allow a better exploration of DRMs in biological compartments, and further studies are needed to define the interest of detecting LFVs in CSF and proviral DNA. This study shows also the global robustness of the UDS in the case of low-VL. To overcome the variability associated to this type of sample as well as proviral DNA, repeated extractions and sequencing may increase the fidelity of the DRMs distribution.

## AUTHOR CONTRIBUTIONS

Cédric Hartard and Evelyne Schvoerer contributed to the study conception and design. Alizée Foury and Cédric Hartard drafted the manuscript. Alizée Foury collected samples and information. Alizée Foury, Cédric Hartard, Aline Saunier, Audrey Taverniers, Nathalie Pinet, and Thomas Josse performed data analysis and investigation. Eliette Jeanmaire and Caroline Emilie took care of the patients and provided clinical data. All authors revised the manuscript, contributed to the article, and approved the submitted version of the manuscript.

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## CONFLICT OF INTEREST STATEMENT

The authors declare no conflict of interest.

## DATA AVAILABILITY STATEMENT

The data that support the findings of this study are available on request from the corresponding author. The data are not publicly available due to privacy or ethical restrictions.

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