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No HIV-1 molecular evolution on long-term antiretroviral therapy initiated during primary HIV-1 infection

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1 **No HIV-1 Molecular Evolution On Long Term Antiretroviral Therapy Initiated During**
2 **Primary HIV-1 Infection.**

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4 **Running head: Viral Diversity In Primary HIV infection**

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44 **Introduction**

45 Primary-HIV1 (PHI) infection is the initial phase of infection. It represents the time when the

46 virus is first disseminating throughout the body and induces host immune responses [1–3]

47 The HIV reservoir is established very rapidly during this stage, due to the provirus integrated

48 into the genome of cells that enables the persistence and the establishment of a latent reservoir

49 which remains the major obstacle to eliminate the virus [4].

50 Several studies have demonstrated that early antiretroviral therapy (ART) initiation can be

51 particularly effective for long term control of HIV-1 replication and it is associated with a faster

52 decay of the latent reservoir, a restriction of its size and an optimal immune restoration [5–7].

53 Whilst, large clinical trials demonstrated that the benefits of earlier treatment mentioned above

54 were limited at these specific criteria because treatment interruption is usually followed by rapid

55 viral rebound, CD4+ T-cells loss and increased risk of morbidity and mortality [8–11].

56 In case of clinical effective ART, residual viremia has been evidenced and could be caused by

57 ongoing low-level virus replication or by release of viral particles and/or viral genome from

58 infected cells [12]. Effectively, it has been suggested that persistent virus replication at low

59 levels may be an important contributor to the maintenance of the reservoir particularly in

60 lymphoid tissue sanctuary sites [13]. However, other studies suggest that there is no evidence
61 of HIV replication on ART and the lack of genetic structure of HIV populations during ART
62 argues against ongoing residual replication [13–15].

63 A better understanding of changes in HIV-1 population genetics with ART is necessary to
64 conclude between these two hypotheses and then for designing and optimizing new eradication
65 strategies. PHI is a particular situation where the low diversity of the transmitted viruses has
66 been described and thus investigating patients who started ART during the earliest stages of
67 their HIV infection provides an opportunity to detect evidence of viral evolution.

68 We aim to study HIV-1 diversity in patients diagnosed with a primary HIV-1 infection, treated
69 at the time of acute infection and with strict effective long term suppressive ART.

70

71 **Materials and methods**

72 We analyzed HIV-1 genetic variation and divergence of viral populations over time in plasma
73 samples (RNA) before ART initiation and in blood cell samples (DNA) during 5 years of viral
74 suppression on ART.

75

76 **Patients**

77 We retrospectively studied 20 patients diagnosed during PHI in the Departments of Infectious
78 Diseases and Internal Medicine at Pitié-Salpêtrière Hospital (Paris, France). PHI was defined

79 as detectable plasma HIV-RNA and an incomplete HIV-1 Western blot, irrespective of the
80 ELISA result (positive or negative) and p24 antigenaemia (positive or negative).

81 Patients received antiretroviral therapy very early after infection and were identified with a
82 strict viral suppression (HIV plasma RNA < 20 copies/mL without any blips) for at least 5 years
83 afterward under ART. Analyses were performed retrospectively on frozen samples of whole
84 blood taken during 5 years of standard follow-up.

85

86 **Ethical considerations**

87 This retrospective study was conducted in accordance with Good Clinical Practices and the
88 ethical principles of the Helsinki declaration, and following ANRS standard practices for
89 clinical research. Patients had written consent that the remnant of their samples could be used
90 for research purpose.

91

92 **Total HIV-DNA quantification**

93 Cell-associated HIV-1 DNA was quantified by ultrasensitive real-time PCR (Generic HIV-
94 DNA assay, Biocentric, Bandol, France) as previously described [16].

95

96 **HIV-RNA quantification and Ultra-Sensitive Viral Load**

97 Plasma Viral Load (pVL) was quantified using the Cobas AmpliPrep/CobasTaqMan HIV-1
98 assay (Roche Diagnostics; lower detection limit of 20 copies/mL). Ultra-sensitive viral load
99 (USVL) in the range of 1–20 copies/mL was indicated qualitatively (presence or absence of
100 detectable signal).

101

102 **Ultra-Deep-Sequencing**

103 **RNA and DNA extraction, amplification and ultra-deep sequencing**

104 HIV RNA was extracted from 1 ml of plasma using easyMAG® (bioMérieux Clinical
105 Diagnostics, France) and HIV DNA was extracted from 400 mL of PBMC using the MagnaPure
106 LC DNA Isolation Kit from Roche according to the manufacturer's instructions.

107 UDS was performed using Illumina Miseq technology (Illumina, San Diego, CA, USA). We
108 deep sequenced 2 fragments RT1 and RT2 of reverse transcriptase (RT) gene (413pb: 2618-
109 3031 and 446pb: 2877-3323 relative to HXB2 genome, respectively) and C2V3 region of gp
110 120 gene (367pb: 7011-7378 relative to HXB2 genome). Details of primers and PCR
111 procedures used for UDS are described in Supplemental Data.

112 Three samples were sequenced per patient: one RNA-HIV: plasma at baseline prior ART
113 initiation, two HIV DNA associated with peripheral blood cells under effective ART: first point
114 of pVL < 20 copies/mL and 5 years follow-up period. Library construction from purified PCR
115 products (RT1, RT2 and C2V3 amplicons) and 2x300 bp Illumina Miseq paired-end sequencing
116 were performed at the Genotyping and Sequencing Platform, ICM Brain and Spine Institute
117 (Paris, France). Sequences were demultiplexed automatically on the MiSeq platform as part of
118 the data processing steps and two paired fastq files were generated for each sample representing
119 the two paired-end reads.

120

121 **HIV drug resistance testing by UDS**

122 To identify Drug Resistance Associated Mutations (DRAMs), the sequence reads were
123 analyzed with IDNS[®] © SmartGene 2019 (Advanced sequencing platform) and resistance
124 (Cutoff detection of minority resistant variant of UDS sequences =2%) was interpreted using
125 the latest ANRS resistance algorithm (<http://www.hivfrenchresistance.org>). Variants present in
126 more than 20% of the quasispecies were considered to be majority resistant variants and variants
127 present at a proportion between 2% and 20% were considered to be minority variants.

128

129 **Phylogenetic analysis**

130 We used Geneious research software (version 11) for phylogenetic analysis [17]. The paired-
131 end reads are merged and quality-filtered to remove noise. Alignment is performed using a
132 target-specific profile and a consensus is produced based on a user-selected ambiguity
133 threshold. We used PhyML for maximum-likelihood (ML) phylogenetic reconstruction
134 (Generalized time-reversible model (GTR)). The best subtree pruning and regrafting (SPR) and
135 the nearest neighbor interchange (NNI) heuristic options were selected. The reliability of tree
136 topologies was assessed by bootstrapping using 1000 replications (values $\geq 70\%$ were
137 considered significant). ML trees were rooted on an outgroup: HIV subtype B consensus
138 (HxB2: K03455; www.hiv.lanl.gov). Tree figures were viewed and modified with FigTree
139 software (<http://tree.bio.ed.ac.uk/software/figtree/>).

140

141 **Diversity analysis**

142 To look for evidence of ongoing viral replication during ART, HIV populations in samples
143 taken at baseline were compared with the populations present during and after long-term ART
144 in RT and gp120 genes. Phylogenetic trees were constructed for each patient with sequences

145 obtained from plasma at baseline prior ART initiation and HIV DNA associated with peripheral
146 blood cells under effective ART (first point of pVL < 20 copies/mL and 5 years follow-up
147 period). Population genetic diversity was calculated as average pairwise difference (APD) using
148 MEGAX (<http://www.megasoftware.net>) [18].

149 Viral evolution was established when temporal structure on ML maximum-likelihood
150 phylogenetic trees and significant change over time of HIV-1 genetic diversity (APD) were
151 observed.

152

153 **HIV-1 Tropism**

154 HIV-1 co-receptor usage was predicted by a genotypic method that used the
155 Geno2phenoreceptor rule (<https://coreceptor.geno2pheno.org/>) and according to ANRS rules
156 (<http://www.hivfrenchresistance.org/hiv-tropism.html>).

157

158 **Statistical analysis**

159 All reported values are medians with interquartile range (IQR) for continuous variables and
160 frequencies and percentages for categorical variables. Changes in cell associated HIV-DNA
161 and CD4 cell count were compared between baseline and month 60 using paired Wilcoxon test
162 and the Mann-Whitney test was for comparison between participants with DRAMs dynamics
163 and those with not. Univariable model was used to identify factors associated with DRAMs:
164 age, sex, transmission group, duration of ART, baseline CD4 and CD8 cell counts, CD4/CD8
165 ratio, nadir CD4 cell count, time since HIV diagnosis, duration of suppressed viraemia, time to
166 ART initiation, time to undetectable viral load under ART, duration of infection, pre-ART pVL,

167 peak of pVL, baseline HIV cell-associated DNA, HIV-1 subtype, CD4 cell count and HIV cell-
168 associated DNA during follow up.

169 All reported P values are two-tailed, with significance set at 0.05. Analyses were performed
170 with SPSS statistics version 23.0 for Windows.

171

172 **Results**

173

174 **Patients' characteristics at time of PHI**

175 Twenty patients with strict viral suppression (HIV viral load <20 copies/mL without any blips)
176 were included in the study with a median age of 47 years (IQR 34-53). Eighteen (90%) were
177 male. Nine of the patients (45%) and 11/20 (55%) were diagnosed at Fiebig stages III and IV,
178 respectively. Fifteen patients (75%) were symptomatic at time of PHI. Time to ART initiation,
179 time to undetectable viral load under ART and duration of infection was in median 5 days (IQR
180 1-12), 95 days (IQR 40-119) and 6 years (IQR 6-7), respectively. Of the 20 patients, 12 (60%)
181 were infected with the clade B virus. Main characteristics of patients are summarized in
182 table 1.

183

184 **Evolution of immunological and virological parameters**

185 Evolution of immunological and virological parameters is presented in figure 1. A median of
186 10 longitudinal plasma HIV-1 RNA was evaluated per patient (197 samples in total). The
187 median HIV-RNA viral load (VL) at time of PHI was 5.7 log₁₀ copies/mL (IQR: 4.94-6.26) and
188 decreased quickly with all patients reaching VL < 20 copies/mL in a median of 95 days (IQR:

189 40 -119) of ART initiation. Ultrasensitive VL (presence of detectable signal between 1-20
190 copies/mL) was found at least once during the 5 years follow-up in 13/20 patients (65%).

191 The total cell-associated HIV-1 DNA level was assayed for a median of 8 longitudinal blood
192 samples per participant (158 samples in total). The median HIV-1 DNA load at PHI and over 5
193 years of follow up was 3.24 log₁₀ copies/10⁶ cells (IQR: 2.72 to 3.49) and 1.60 log₁₀ copies/10⁶
194 cells (IQR:1.60), respectively. The analysis revealed a global and significant decrease in total
195 HIV-1 DNA during follow up period (p= 0.02). The total cell-associated HIV-1 DNA load was
196 not detectable (<40 copies/10⁶ cells) for 14 (70%) patients at the end of follow up.

197

198 **Resistance analysis and tropism**

199 At baseline, DRAMs were detected in RT gene in 3 (15%) patients with 2 majority resistant
200 variants: 2 K103N (98%) and 1 minority resistant variant Y188H (2%). Regarding longitudinal
201 dynamics of NRTI and NNRTI DRAMs, DRAMs at baseline were compared with archived
202 DRAMs during follow up period (table 2). New DRAMs were detected in 9 patients (45%)
203 despite fully sustained suppression of HIV-RNA in plasma with new archived DRAMs in 4/20
204 (25%) patients at the first point of pVL < 20 copies/mL: patients 4, 5, 16 and 17. Seven (35%)
205 individuals had at least 1 emerging DRAM in peripheral blood cells after 5 years of follow up
206 (most of them were detected at less than 10%): patients 3, 4, 9, 10, 11, 12 and 16 (table 2).
207 Fifty-five percent (5/9) of patients who showed emergence of DRAMs had one residual viremia
208 at least once during follow up. The comparison between the characteristics of patients with new
209 archived DRAMs and those without revealing that only transmission group is associated with
210 the dynamics of archived DRAMs. Effectively, all patients who had variants with new DRAMs
211 were MSM (P= 0.02).

212 Five patients (25%) had at least one G-to-A mutation resistance associated mutation: patient 4:
213 D67N (9.9%) and M184I (2.7%); patient 5: M230I (98.9%); patient 10: M184I (2.3%); patient
214 12: D67N (7.2%) and patient 16: D67N (4.4%), M184I (98%) and M230I (100%) (table 2).

215 Fifteen patients (75%) harbored a CCR5-tropic virus. The genotypic prediction of C2V3 co-
216 receptor tropism did not vary over time in all patients.

217

218 **Search of potential HIV genetic evolution - Phylogenetic studies**

219 Phylogenetic analyses were processed in the 20 individuals, in RT and gp120 gene. Analysis
220 showed that in all patients, sequences were intermingled: in each patient, sequences obtained
221 from three different time points were highly homogenous. Tree topologies showed an absence
222 of segregation between sequences in HIV-1 RNA at baseline prior to ART and in cell-associated
223 HIV-DNA during 5 years of ART (figure 2).

224 The average nucleotide pairwise distance (APD) was estimated between the reads obtained
225 from each time point. In the first sample (in HIV-RNA at baseline prior to ART), the median
226 of APD was 1% (IQR: 1-1), 1% (IQR: 1-1) and 2% (IQR: 1-2) in RT1 fragment, RT2 fragment
227 and gp120 gene respectively. In the second sample (in cell associated HIV-DNA; first time
228 point of plasma VL < 20 copies/mL) the median of APD was 1% (IQR: 1-2), 1% (IQR: 1-1)
229 and 2% (IQR: 1-3.75) respectively in RT1 fragment, RT2 fragment and gp120. In the third
230 sample (in cell associated HIV-DNA after 5 years of follow up), the median of APD was 1%
231 (IQR: 1-2), 1% (IQR: 1-1) and 2% (IQR: 1-2) in RT1 fragment, RT2 fragment and gp120 gene
232 respectively. This comparison of the APD in sequences obtained from samples taken at different
233 times showed the absence of arguments of significant viral diversity evolution between primary
234 infection and during the following 5 years.

235

236 **Discussion**

237 To our knowledge, it is the first study to report quantitative and qualitative analysis by UDS of
238 HIV-1 reservoir in twenty patients diagnosed in the acute phase of infection, treated very early
239 and had strict effective long term suppressive ART (during at least 5 years of follow up). There
240 was a significant decay of HIV-RNA and cell-associated HIV DNA in our study in participants
241 who started ART during the first month of their infection. Phylogenetic analysis showed the
242 absence of genetic divergence and diversity in the RT and gp120 genes over time. However,
243 despite sustained virological control under ART, some minor variations (emergence or
244 disappearance) of DRAMS were evidenced associated or not with the current antiretroviral
245 treatment.

246 Our results are in line with a number of studies showing the faster decrease of HIV DNA in
247 patients starting ART during acute HIV-1 [19–21] infection . However, some clinical trials
248 evidenced a viral rebound after prolonged virologic suppression with no difference in
249 virological and immunological parameters between immediate and delayed treatment in the
250 vast majority of cases [8–11,22].

251 In this study, 15% of NNRTI DRAMs (2 K103N and 1 Y188H) were revealed at the time of
252 primary HIV infection. This prevalence is similar to the latest data (2014-2016) from the French
253 cohort of primary infections showing that 18.6% of patients had DRAMS at baseline with the
254 highest level of resistance to the NNRTI class (13.4%) [23].

255 Concerning longitudinal dynamics of minority variants, new DRAMs, not detected at baseline,
256 appeared during follow-up in some patients treated early during the acute phase of HIV-1
257 infection despite a fully controlled viremia < 20 cp/mL and absence of detected residual
258 viremia in 45% (4/9) of cases. This finding is consistent with the assessments carried out by

259 Gantner et al; they used the UDS technique to assess the longitudinal dynamics of viral resistant
260 quasispecies archived in blood and demonstrated that, despite virological control, the diversity
261 of the quasispecies continued to evolve [24]. This could be the result of persistence of a residual
262 viremia below the limit of standard quantification in some patients. Other researchers suggested
263 that the new variants have most probably been selected directly in the blood compartment or
264 other reservoirs because of insufficient drug penetration [24–26]. In addition, some of these
265 new DRAMs in blood cells were G-to-A mutations implicating APOBEC3 editing, a cellular
266 enzyme action and not viral replication [27].

267 The results of phylogenetic analysis, as well phylogenetic tree and average pairwise distance,
268 suggested the absence of genetic changes in archived HIV-1 DNA in our patients treated during
269 the acute phase of infection with effective ART. Our findings support the majority of the studies
270 reporting the lack of viral evolution during suppressive ART in chronically HIV infected adults,
271 as well as in children treated shortly after birth when viral diversity is low [14,15,28]. This
272 absence of sequence divergence is indicative of long-lived cells infected and argues against
273 viral replication being the major source of persistent viremia [14,15,28–30]. On another side,
274 Lorenzo *et al.* reported that anatomical sanctuary sites such as the lymph nodes can allow
275 residual viral replication on ART, contributing to the maintenance of the HIV reservoir [31].
276 These findings were strongly criticized by Kearney *et al.* who re-analyzed data and reported
277 limits of data according to the low number of samples, the short time of the survey and absence
278 of evidence of viral evolution using more complex analyses [29]. There are several reasons to
279 explain controversy results such as differences in study populations (children treated shortly
280 after birth, adults with chronic infection, patients diagnosed in acute phase of infection), in
281 sampling (plasma, cell associated HIV-DNA, lymph nodes), in sequencing (Sanger sequencing,
282 Single Genome Sequencing, UDS: 454 Roche and Illumina technology) and in analysis

283 (phylogenetic analysis, measure of the APD, test of panmixia and others mathematics
284 methods...) [13–15,28–30,32].

285 Phylogenetic analysis allowed the comparison of the integrality of sequences which is more
286 informative about viral diversity than the study of few resistance positions in the RT sequence.
287 Then, the impact of rare resistant variants could be diluted and doesn't impact phylogenetic
288 analysis and could explain the apparent discrepancy between the DRAMs variation and the
289 stability in phylogenetic analysis in our study.

290

291 **Conclusion**

292 In conclusion, this study is the first to use the UDS technique to assess the longitudinal
293 dynamics of viral populations in plasma prior to ART and in archived blood cells in sustained
294 ART in patients diagnosed with a primary HIV-1 infection and treated very early. Despite a
295 slight variation of minority resistance-associated mutation variants, there was no clear evidence
296 of viral evolution during a prolonged period of time. Our results underlined that ART initiation
297 during PHI is fundamental to positively impact quantitative and qualitative biological
298 parameters related to the HIV-1 reservoir in order to reduce its size and to control the viral
299 diversity in the perspective to design of HIV-1 cure strategies.

300

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303

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305 All other authors: none to declare.

306

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311 wrote the paper; All authors reviewed and accepted the final version of the manuscript.

312

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Characteristics	Total= 20
Age (years), median (IQR)	47 (34 - 53)
Sex- n (%)	
-Male	18 (90 %)
-Female	2 (10%)
Origin- n (%)	
-Caucasian	19 (95%)
-Others	1 (5%)
Transmission group- n (%)	
-Men who have Sex with Men	15 (75%)
-Heterosexual	5 (25%)
Symptomatic PHI- n (%)	15 (75%)
HLA B57*01- n (%)	
Negative	10 (50%)
ND	10 (50%)
Fiebig stage	
III	9 (45%)
IV	11 (55%)
HIV-1 RNA Zenith (log ₁₀ copies/mL), median (IQR)	5.82 (4.94-6.26)
HIV-1 RNA (log ₁₀ copies/mL), median (IQR)	5.7 (4.94-6.26)
HIV DNA (log ₁₀ copies/10 ⁶ cells), median (IQR)	3.24 (2.72-3.49)
CD4 nadir cell count (cells/mm ³), median (IQR)	417 (325-522)
CD4 cell count (cells/ mm ³), median (IQR)	483 (325-566)
Duration of infection (years), median (IQR)	6 (6-7)
Time from diagnosis to ART initiation (days), median (IQR)	5 (1-12)
Time to undetectable viral load (days), median (IQR)	95 (40 -119)
ART regimen n (%)	
-2 NRTI+2 PI	8 (40%)
-2 NRTI + 1 INI	8 (40%)
-2 NRTI + 1 NNRTI	2 (10%)
-2 NRTI+2 PI+MVC	1 (5%)
-2 NRTI + MVC	1 (5%)

Table 1 : Patients' characteristics at the time of primary infection

PHI=primary HIV-1 infection; ND: Not Determined; NRTI=nucleoside reverse transcriptase inhibitor; NNRTI= non-nucleoside reverse transcriptase inhibitor; PI= protease inhibitor; MVC=maraviroc;

Patient	Time point	Ongoing ART	NRTI DRAMs (% quasispecies)	NNRTI DRAMs (% quasispecies)
3	RNA DNA*	TDF/FTC/DRV/RTV TDF/FTC/RPV		Y188H (2.0%)
	DNA**	TDF/FTC/DTG	<i>M41L (9.1%), T69S (13.6%), L74V (6.0%)</i>	
4	RNA DNA*	ABC/3TC/RAL		<i>K103N (5,5%)</i>
	DNA**	TDF/FTC/RPV	D67N (9.9%), K70R (2.4%), M184I (2,7%), M184V (4.8%)	<i>V90I (2,2%), K103N (7,1%), E138A (2,7%)</i>
<u>5</u>	RNA DNA* DNA**	TDF/FTC/DRV/RTV TDF/FTC/DRV/RTV	M230I (98.9%)	
<u>9</u>	RNA DNA* DNA**	TDF/FTC/DRV/RTV TDF/FTC/RPV	<u>M184V (8.8%)</u>	<u>Y188L (2%)</u>
<u>10</u>	RNA DNA* DNA**	TDF/FTC/DRV/RTV TDF/FTC/RPV	M184I (2.3%), M184V (4.5%)	<i>G190V (2.1%)</i>
11	RNA DNA*	TDF/FTC/MVC		
	DNA**	TDF/FTC/RPV TDF/FTC/DTG 3TC/DTG		<u>V179I (10.2%), V179D (2.3%)</u>
<u>12</u>	RNA DNA*	TDF/FTC/RAL TDF/FTC/RPV		
	DNA**	TAF/FTC/EVG/COB	<i>M41L (3.2%), D67N (7.2%)</i>	<i>K103N (7.9%)</i>
<u>16</u>	RNA DNA*	TDF/FTC/RAL		E138A (45.4%)
	DNA**	TDF/FTC/RPV 3TC/DTG	D67N (4.4%), M184I (98.0%), M184V (2.0%)	M230I (100.0%)
17	RNA DNA*	TDF/FTC/DRV/RTV/MVC	<i>M41L (7.8%), T69N (3.7%), T69S (4.9%)</i>	<i>K103N (11.6%)</i>
	DNA**	TDF/FTC/DRV/RTV TDF/FTC/EFV		

Table 1 : UDS evolution of archived Drug Resistance Associated Mutations in plasma (RNA) at baseline and cell-associated HIV-1 DNA (DNA) under effective antiretroviral

Therapy: results in % (mutation frequency among all reads); ; NRTI=nucleoside reverse transcriptase inhibitor; NNRTI= non-nucleoside reverse transcriptase inhibitor; 3TC: lamivudine; ABC: abacavir; TDF: tenofovir disoproxil fumarate; FTC: emtricitabine; RPV: rilpivirine; EFV: efavirenz; DRV/RTV: darunavir/ritonavir; RAL: raltegravir; DTG: dolutegravir, EVG: elvitégravir;; MVC, maraviroc; COB:cobicistat

DNA*: first point of plasma viral load < 20 copies/mL, DNA**: 5 years of follow-up period

Underlined: mutations associated with prescribed treatment; *Italic*: mutations not related with prescribed treatment; **Bold**: G-to-A mutations; **Bold and Underlined**: patients with at least 1 residual vireamia during follow up.

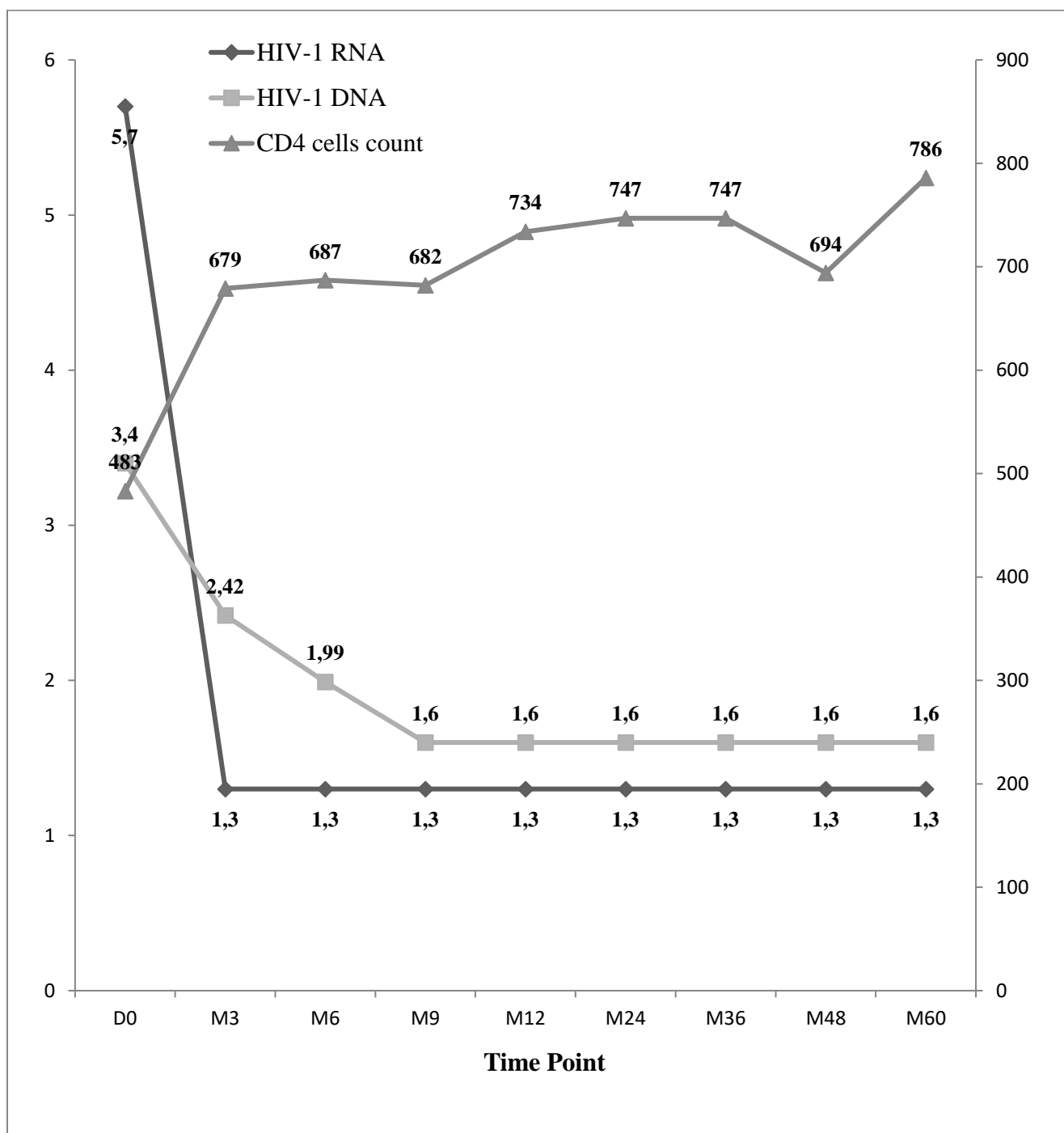


Figure 1: Median of longitudinal plasma HIV-1 RNA, cell associated HIV-1 DNA levels and CD4 cells count in 20 patients: All available samples for 5 years in 20 patients are displayed; D0= Day with confirmed diagnosis of primary HIV-1 infection; M=Month; HIV-1 RNA (Log₁₀ copies/mL); HIV-1 DNA (Log₁₀ copies/10⁶ cells); CD4 cells count (cells/mm³)

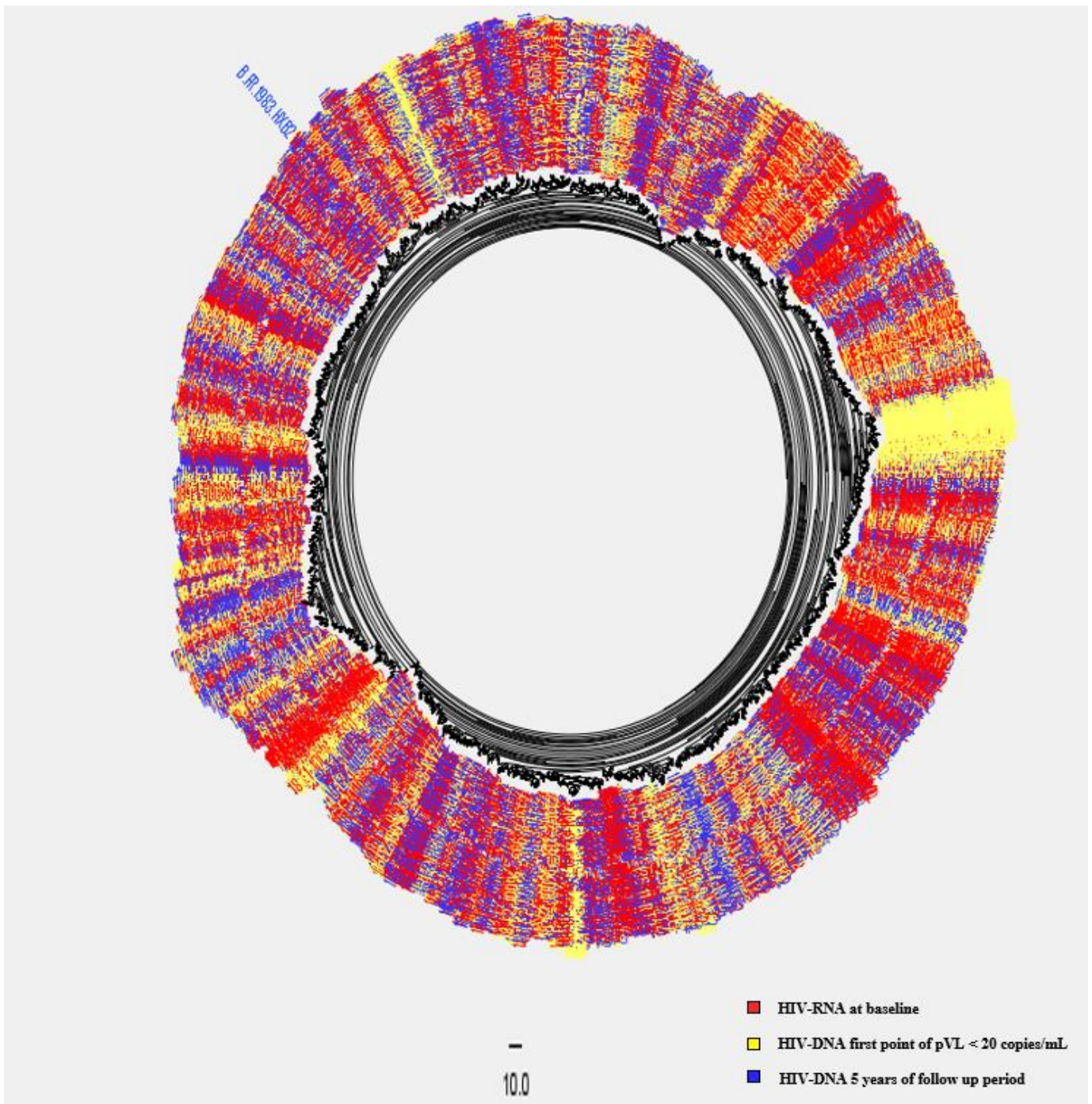


Figure 1 : Example of phylogenetic tree for patient 18 constructed from HIV-RNA at baseline prior ART initiation and HIV-DNA associated with peripheral blood sequences: We used PhyML for maximum-likelihood (ML) phylogenetic reconstruction (Generalised time-reverse model (GTR)); ML tree was rooted on an outgroup: HIV subtype B consensus (HxB2: K03455); phylogenetic tree showing intermingled sequences in RT gene.

Supplemental Data

We deep sequenced RT gene into 2 fragments: RT1 (413pb), RT2 (446pb) and gp120 gene (367pb). These genes were amplified using two rounds of PCR amplification.

Details of primers used for amplification are listed in table 1.

PCR1 Forward (for) and PCR1 Reverse (rev) for PCR round one, and PCR 2 for and PCR2 rev for PCR round 2 (nested PCR). To amplify RT1 and RT2 fragments, the following thermocycler parameters were used for PCR1: 50 °C for 30 minutes (mn), 94 °C for 7 mn, 94 °C for 10 seconds (s), 55 °C for 30s, 68 °C for 1 mn, 35 cycles of steps 3–5 and 68 °C for 7 mn. We used a touchdown PCR for round 2 with the following parameters: 98 °C for 1 mn, 3cycles (98°C for 10s; 66-64 °C for 30s; 72°C for 15s), 3cycles (98°C for 10s; 64-62 °C for 30s; 72°C for 15s), 3cycles (98°C for 10s; 62-60 °C for 30s; 72°C for 15s), 30 cycles (98°C for 10s; 60 °C for 30s; 72°C for 15s), and 72 °C for 7 mn.

To amplify ENV C2V3 region, the following thermocycler parameters were used for PCR1: 50 °C for 30 minutes (mn), 94 °C for 7 mn, 94 °C for 10 seconds (s), 53 °C for 30s,

68 °C for 1 mn, 35 cycles of steps 3–5 and 68 °C for 7 mn. Parameters used for PCR2 were:

98 °C for 1 mn, 98°C for 10s, 60 °C for 30s; 72°C for 15s, 40 cycles of steps 2-4 and 72 °C for 7 mn.

Primer Name	Primer Sequence (5'-3' orientation)
RT1-PCR1-for	TAGTCCTATTGARACTGTACCAGT
RT1- PCR1-rev	ATCCTACATACAARTCATCCATG
RT1- PCR2-for	ATGGCCATTGACAGAAGAAA
RT1- PCR2-rev	TGGAATATTGCTGGTGATCC
RT2- PCR1-for	GGGARGTYAATTAGGAATACC
RT2- PCR1-rev	AGTCTTTTGATGGGTCATAATA
RT2- PCR2-for	GATGTGGGkGATGCATATTT
RT2- PCR2-rev	CTGTATGTCATTGACAGTCCAG
V3- PCR1-for	CAG TAC AAT GTA CAC ATG G
V3- PCR1-rev	ATG GGA GGG GCA TAC ATT G
V3- PCR2-for	TTACAGTAGAAAAAT TCC CCT C
V3- PCR2-rev	AAT GGC AGTCTA GCAGAA G

Table 1 : Primers used for Ultra Deep Sequencing

for=forward; rev=reverse