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Increasing contribution of integrated forms to total HIV DNA in blood during HIV disease progression from primary infection

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32

33 **Abstract**

34 **Background:** In the current context of research on HIV reservoirs, offering new insights into
35 the persistence of HIV DNA in infected cells, which prevents viral eradication, may aid in
36 identifying cure strategies. This study aimed to describe the establishment of stable integrated
37 forms among total HIV DNA during primary infection (PHI) and their dynamics during the
38 natural history of infection.

39 **Methods.** Total and integrated HIV DNA were quantified in blood from 74 PHI patients and
40 97 recent seroconverters (<12 months following infection, “progression cohort”). The
41 evolution of both markers over six years were modelled (mixed-effect linear models). Their
42 predictive values for disease progression were studied (Cox models).

43 **Findings.** For most patients during PHI, stable integrated forms were a minority among total
44 HIV DNA (median: 12%) and became predominant thereafter (median at AIDS stage: 100%).
45 Both total and integrated HIV DNA increased over a six-year period. Patients from the
46 progression cohort who reached clinical AIDS during follow-up (n=34) exhibited higher total
47 and integrated HIV DNA levels at seroconversion and a higher percentage of integrated forms
48 than did slower progressors (n=63) (median: 100% vs 44%). The integrated HIV-DNA load
49 was strongly associated with the risk of developing AIDS (aRR=2.63, $p=0.002$).

50 **Interpretation.** The profile of “rapid” or “slower” progression in the natural history of HIV
51 infection appears to be determined early in the course of HIV infection. The strong
52 predominance of unstable unintegrated forms in PHI may explain the great benefit of this
53 early treatment, which induces a sharp decrease in total HIV DNA.

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55

56 **Keywords**

57 Total HIV DNA; integrated HIV DNA; reservoirs; natural history; primary HIV infection;

58 acquired immunodeficiency syndrome; kinetics

59

60 **Introduction**

61

62 Combined antiretroviral therapy (cART) has constituted major progress in the treatment of
63 HIV infection and current recommendations are to treat all HIV-infected persons, regardless
64 of the stage of infection. Although cART can efficiently block HIV replication, it cannot
65 completely eliminate the virus from its reservoirs. In particular, infected resting CD4+ T-cells
66 containing latent integrated HIV proviruses are refractory to current cART and represent a
67 major hurdle preventing viral eradication ^{1,2}. Understanding the pathogenesis of HIV
68 reservoirs is critical for developing and evaluating new therapeutic strategies aimed at viral
69 eradication or functional cure.

70

71 Integrated HIV DNA is the most stable and functional form of the viral genome. It plays a
72 major role in the pathogenesis of HIV infection and HIV reservoirs, even if it includes both
73 replication-competent and defective genomes ³⁻⁵. Cells containing integrated HIV DNA can
74 produce new infectious virions upon stimulation and activation ^{6,7}. Proviruses persist
75 indefinitely, partly due to the homeostatic proliferation of memory T-cells. In contrast,
76 unintegrated forms, which include linear and episomal HIV DNA with 1- or 2-LTRs, are
77 considered more labile and are surrogate markers of viral replication ^{8,9}.

78 Total HIV DNA levels in peripheral blood mononuclear cells (PBMCs) have been described
79 during both the natural history of HIV infection and cART ¹⁰. It remains partially unexplained
80 why the decrease in this surrogate marker of the reservoir is greater and faster when treatment
81 is initiated during primary HIV infection (PHI) than during the chronic stage ¹¹⁻¹³. As for
82 integrated HIV DNA, it has been mostly studied in patients on cART ¹³⁻¹⁵. Only a few studies
83 have measured integrated HIV DNA in untreated patients, most of which include fewer than
84 20 patients ^{14,16-18}. Longitudinal data are even more scarce for both markers: only one study to

85 our knowledge has a follow-up of patients during untreated infection (17 patients during the
86 first year, among which 10 still followed after 2-8 years)¹⁸. Moreover, some techniques do
87 not allow a comparison between the amounts of total and integrated HIV DNA because of
88 differences in assay standardization¹⁹ and the long-term dynamics of integrated forms among
89 total HIV DNA have never been described. Besides, all previous results showed a great inter-
90 individual variability of total and integrated HIV DNA^{14,16,17,20,21}, enhancing the need for
91 studies on large cohorts.

92

93 To better understand the establishment and maintenance of the HIV reservoir in the blood, the
94 objectives of this work were i) to describe the contribution of integrated forms to total HIV
95 DNA in untreated patients at different stages from PHI to chronic infection and ii) to evaluate
96 the predictive value of these biomarkers at the time of seroconversion with respect to
97 evolution towards clinical AIDS.

98

99 **Materials and Methods**

100

101 **Study population**

102 Patients with a known or estimated date of infection from two ANRS French cohorts were
103 selected. The ANRS-PRIMO cohort was approved by the Ile-de-France-3 Ethics Committee
104 (CPP-1157). All patients from both cohorts gave written informed consent.

105 The ongoing ANRS-PRIMO CO6 cohort enrolls patients presenting with PHI, as previously
106 described^{12,22}. All patients are treatment-naïve at inclusion. Patients enrolled between June
107 2015 and April 2016 with available frozen PBMCs samples from the time of their inclusion
108 were selected for this study.

109 In the ANRS-SEROCO CO2 cohort, initiated in 1988, recently diagnosed HIV-infected
110 individuals who were free of AIDS-related diseases at inclusion were enrolled ²³. Follow-up
111 was scheduled every six months until 2009. Patients were selected within this cohort
112 according to the following criteria: enrolment within 12 months following infection, no
113 efficient cART received before inclusion and during the study until 1996, and having at least
114 two frozen PBMCs samples available.

115 In both cohorts, the infection date was defined as the date of the incomplete Western blot
116 minus one month, or the date of a primary symptomatic infection minus 15 days, or the
117 midpoint between a negative and a positive HIV antibody test.

118

119 **Quantification of total HIV DNA and integrated HIV DNA and determination of** 120 **unintegrated HIV DNA**

121 Total HIV DNA and integrated HIV DNA were quantified in all PBMCs samples. Total DNA
122 was extracted with the QIAamp DNA Blood Mini Kit (Qiagen, France) according to the
123 manufacturer's instructions.

124 First, total HIV DNA was quantified by real-time PCR with the Generic HIV DNA CELL kit
125 (Biocentric, France), employing the 8E5 line as the standard ³. Based on a previously reported
126 technique ²⁴, we developed a nested *Alu*-LTR PCR assay to quantify the integrated HIV DNA
127 from the same extracts as total HIV DNA, using the HeLaR7Neo cell-line as the standard with
128 improved sensitivity and reproducibility (Supplementary Text 1). The quantification threshold
129 varied depending on the number of available cells tested (threshold range [5-97] copies/10⁶
130 PBMCs (0.70-1.97 log₁₀)). The equivalence of HIV DNA copies between HeLaR7Neo and
131 8E5 standards was verified, allowing the determination of the percentage of integrated forms
132 among total HIV DNA. Unintegrated HIV DNA levels were calculated by the difference in
133 the number of copies between total and integrated HIV DNA.

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Statistical analyses

Statistical analyses were performed using Stata software (v14.2, StataCorp, USA) and Prism software (v7, GraphPad, USA). Continuous variables were compared between groups by using Wilcoxon tests and qualitative variables with Fisher's exact tests. Spearman's correlation coefficient was employed to quantify the correlations between continuous variables. Cox models were used to determine the predictive values of HIV DNA levels for clinical AIDS onset. Mixed-effect linear models (MELMs) were employed to estimate changes in total HIV DNA and integrated HIV DNA over time, considering the value at enrolment as time t=0 for each patient. Logarithm₁₀ transformation of HIV RNA and HIV DNA levels was performed to fulfil the model assumptions. Integrated HIV DNA levels were set to half of the threshold when undetectable. To evaluate the robustness of our results, additional analyses were performed, setting the values of undetectable integrated HIV DNA levels to the threshold value, to zero, or to a range of randomized values between 1 and the threshold. All these analyses produced results similar to those obtained when the values of undetectable integrated HIV DNA levels were set to half the threshold value. *P* values<0.05 were considered significant.

Results

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153
154

Patient characteristics

Seventy-four patients who presented during PHI (ANRS-PRIMO cohort ²²) were included, with a median [range] estimated time since infection of 1.2 [0.4-2.4] months (Fiebig II-III, n=12; Fiebig IV, n=7; Fiebig V, n=35; Fiebig VI, n=20). Ninety-seven recent seroconverters

158 from the historical ANRS-SEROCO cohort were included (median estimated time between
159 infection and enrolment: 6.2 [1.3-11.9] months) (called later “progression cohort”) ²³. The
160 characteristics of the 171 study patients are presented in Table 1. As expected, the PHI
161 patients had higher HIV RNA loads ($p<0.001$) (see also Supplementary Figure 1) and lower
162 CD4+ T-cell counts than did those from the progression cohort ($p=0.036$).

163

164 Among the progression cohort, the following two groups could be defined regarding
165 subsequent evolution during follow-up: individuals who developed AIDS (rapid progressors,
166 $n=34$) and those who had not developed AIDS by 1996 (slower progressors, $n=63$) (Table 1).
167 Notably the follow-up time was significantly longer for the slower progressors group. Hence,
168 AIDS occurrence in rapid progressors was not due to a longer follow-up time.

169

170 **Low percentage of integrated forms in PHI, followed by an increase in the first year post** 171 **infection**

172 We quantified the amounts of total HIV DNA and integrated HIV DNA in the samples taken
173 at inclusion from all 171 patients. The results were combined to describe the profiles of both
174 markers over the first year following infection (Figure 1a). Loess curves showed a low level
175 of integrated HIV DNA during PHI, which reached higher levels later in the first year
176 following infection, while the level of total HIV DNA was high during PHI and decreased
177 thereafter. Both markers reached a steadier state after 90 days post infection. The percentage
178 of integrated HIV DNA among total HIV DNA was low for patients in the first 90 days after
179 infection (median: 12%), while it became much higher between 3 and 12 months after
180 infection (median: 65%). Accordingly, even though this percentage varied from 0.1 to 100%
181 regardless of the delay between infection and enrolment, the number of patients with a high
182 percentage of integrated HIV DNA increased with increasing time since infection (Figure 1b).

183

184 The median total HIV DNA level in PHI patients was $3.59 \log_{10}$ copies/ 10^6 PBMCs [range:
185 $2.11-5.61 \log_{10}$], which was significantly higher than that in the 97 progression cohort
186 patients ($3.22 [2.14-4.30] \log_{10}$ ($p=0.008$) and $2.96 [1.30-4.00] \log_{10}$ ($p<0.001$) for rapid and
187 slower progressors, respectively) (Table 1, Figure 2a). Conversely, the median integrated HIV
188 DNA levels in PHI patients ($2.15 [0.48-4.10] \log_{10}$ copies/ 10^6 PBMCs) and slower
189 progressors ($2.53 [0.30-3.96] \log_{10}$) did not significantly differ from each other ($p=0.314$) but
190 were significantly lower than those in rapid progressors ($3.32 [0.60-4.11] \log_{10}$) ($p<0.001$)
191 (Table 1, Figure 2b). Among patients from the progression cohort, both total HIV DNA and
192 integrated HIV DNA levels were significantly higher in rapid progressors than in slower
193 progressors (Table 1). Notably, the median percentage of integrated among total HIV DNA
194 was 100% for rapid progressors, while it was 44% for slower progressors, indicating that
195 integrated HIV DNA already represented the major form of HIV DNA in the rapid
196 progressors group.

197

198 Overall total HIV DNA levels were significantly correlated with the integrated HIV DNA
199 (Supplementary Figure 2) and plasma HIV RNA levels (Supplementary Figure 3).
200 Interestingly, for PHI patients, the HIV RNA load showed the strongest correlation with the
201 unintegrated HIV DNA level, a marker of ongoing viral replication, while for patients from
202 the progression cohort at the inclusion time (both rapid and slower progressors), the HIV
203 RNA load was more strongly correlated with the integrated HIV DNA level than with the
204 level of unintegrated forms (Supplementary Figure 4-5).

205

206 **Increase in total HIV DNA and integrated HIV DNA loads during years of untreated**
207 **infection**

208 Among the 97 HIV-infected individuals from the progression cohort, the median time
209 between the first and the last sample was 4.5 years (range: [1.5-7.7]) or 5.8 years [2.9-8.1]
210 for rapid progressors or slower progressors, respectively. HIV DNA kinetics were studied
211 based on a total of 340 samples, with a median of four frozen cell samples per patient (range:
212 [2-6]). Total HIV DNA levels significantly increased over time ($p < 0.001$), showing a $+0.627$
213 \log_{10} (+5371 copies/ 10^6 PBMCs) increase in rapid progressors and a $+0.574$ \log_{10} (+2508
214 copies/ 10^6 PBMCs) increase in slower progressors over six years (Figure 3a); the increases
215 were similar in the two groups ($p = 0.740$). The integrated HIV DNA level also significantly
216 increased over the same six-year period, exhibiting similar increases ($p = 0.300$) in rapid
217 progressors ($+0.538$ \log_{10} (+5122 copies/ 10^6 PBMCs), $p = 0.008$) and slower progressors
218 ($+0.796$ \log_{10} (+1779 copies/ 10^6 PBMCs), $p < 0.001$) (Figure 3b). Over time, slower
219 progressors maintained lower levels of both total HIV DNA and integrated HIV DNA than
220 rapid progressors, while the percentage of integrated forms tended to increase only in slower
221 progressors ($p = 0.056$). During the same six-year period, CD4⁺ T-cell counts decreased in
222 both groups ($p < 0.001$), with a steeper decline in rapid progressors than in slower progressors
223 (-427 and -222 CD4⁺ T-cells/ mm^3 respectively, $p = 0.002$) (Supplementary Figure 6).

224
225 Twenty-four patients among the rapid progressors had an available sample from the 12
226 months before clinical AIDS occurrence or at the AIDS stage. The median total HIV DNA
227 and integrated HIV DNA loads were 3.64 \log_{10} (IQR [3.42-3.98]) and 3.69 \log_{10} [3.31-3.97],
228 respectively. The median percentage of integrated forms was 100% (IQR [82.3-100]).

229

230 **High predictive value of the integrated HIV DNA load for evolution towards clinical**
231 **AIDS**

232 Given the observed differences in the viral parameters at inclusion between the rapid and
233 slower progressors groups, we evaluated their predictive value for evolution towards AIDS.
234 The CD4+ T-cell counts at inclusion were similar between the two groups ($p=0.124$) (Table
235 1) and were not included in the models. Univariate Cox models showed that a one-log
236 increase in the amount of total HIV DNA, integrated HIV DNA, or HIV RNA at the baseline
237 was significantly associated with a 2.48- to 3.12-fold increase in the risk of developing AIDS
238 (Table 2). Since the amounts of total HIV DNA and integrated HIV DNA were strongly
239 linked, two separate multivariate Cox models were applied, with adjustment for sex, age and
240 HIV RNA level at enrolment. In the model including total HIV DNA, a higher HIV RNA load
241 was associated with an increased risk of progression to AIDS (adjusted risk ratio for a one-log
242 positive difference (aRR)=2.02, $p=0.044$) (Table 2). In the second multivariate model, only
243 the integrated HIV DNA load was strongly associated with the risk of progression (aRR=2.63,
244 $p=0.002$) (Table 2).

245

246 **Discussion**

247

248 The presence of long-lived infected cells that are not targeted by cART currently prevents
249 viral eradication and cure. Understanding HIV pathogenesis by generating new insights into
250 the dynamics of the HIV DNA components during infection is critical for conceiving of
251 therapeutic strategies targeting these reservoirs.

252

253 This was the first large study to quantify both total HIV DNA and integrated HIV DNA,
254 which is the main persistent form of HIV ^{25,26}, in samples collected during PHI, chronic
255 infection, and at various time points until the AIDS stage, which was made possible by two
256 large French cohorts. The ANRS-SEROCO cohort is one of the rare large historical cohorts
257 that included patients in the 1990s who, at that time, remained largely untreated during their
258 follow-up and who had available longitudinal frozen cell samples.

259

260 One of the objectives of this study was to explore the link between the amount of stable
261 proviruses and the risk of HIV disease progression, with the hypothesis that unstable
262 unintegrated forms may have a lesser impact on long-term evolution. From the time of recent
263 seroconversion, the amount of total HIV DNA was higher in rapidly progressing patients than
264 in slower progressors, which agreed with its known predictive value for HIV disease
265 progression ^{10,27}. Here, we report for the first time the predictive value of the amount of
266 integrated HIV DNA, which appeared to be even more strongly predictive of the risk of
267 developing clinical AIDS. Integrated HIV DNA is probably mostly responsible for the
268 predictive value of total HIV DNA observed in previous studies, since this stable form of HIV
269 persistence drives the course of infection ^{2,5}. Integrated HIV DNA is indeed the major source
270 of the viral replication and the HIV RNA level reflects the proportion of cells containing this
271 HIV stable form. Yet, the pathogenic impact of integrated HIV DNA is not only through the
272 viral replication since its predictive value is independent of the HIV RNA level. Another
273 potential means to drive the pathogenesis could be the production of transcripts and antigens
274 from defective integrated genomes, which do not result in HIV RNA viremia, but contribute
275 to the immune activation, as previously described ²⁸. The concomitant study of total HIV
276 DNA and integrated HIV DNA showed that the percentage of integrated forms is highly
277 informative. Indeed, this percentage was already high in rapid progressors at a time point

278 within 12 months from seroconversion and remained high during disease progression,
279 confirming that early events determine the profile of a “rapid progressor” or “slower
280 progressor”. Total HIV DNA being strongly predictive of progression towards AIDS in PHI ²⁹,
281 the few PHI patients with a high proportion of integrated forms might be at a particularly high
282 risk of becoming rapid progressors.

283 These data are in line with the very low levels of integrated HIV DNA observed in the unique
284 group of elite suppressors compared to patients under efficient cART with similar total HIV
285 DNA levels ³⁰. The low levels of integrated HIV DNA may be the consequence of more
286 efficient specific cytotoxic T-lymphocyte (CTL) responses. CTLs could have preferentially
287 destroyed cells harbouring integrated viral DNA, in which the viral transcription and
288 translation into proteins is more efficient, eventually leading to the maintenance of lower
289 proportions of integrated forms ³¹. Rapid progressors may have less efficient CTL responses
290 and therefore maintain higher levels of integrated HIV DNA.

291
292 Studies examining total HIV DNA have shown that the establishment of reservoirs is a very
293 early event in PHI ³². Although integrated HIV DNA is included in total HIV DNA,
294 concomitant study of these two markers showed that they exhibit very different kinetics in the
295 first year of infection, where the first three months after infection stand apart from the
296 following months and years. Integrated HIV DNA levels were found to be low for most
297 patients during PHI, while total HIV DNA levels were particularly high at that time, which
298 was consistent with previous data showing a peak during this stage ^{29,32}. We observed a slight
299 short decrease in integrated HIV DNA levels after the first weeks of infection (Fiebig III-IV),
300 which is in agreement with the results previously reported for 19 untreated PHI patients from
301 whom samples were collected during earlier stages (17 at Fiebig I-II stages), which showed a
302 peak of integrated HIV DNA in week 2 and a decrease until week 6 after enrolment ¹⁸.

303 Ananworanich's work presented some results describing the evolution of total HIV DNA and
304 integrated HIV DNA for ten patients during 144 weeks¹⁸. However, only the median results
305 were presented for these two biomarkers and the proportions of stable forms among total HIV
306 DNA and their evolution over time were not detailed for the different individuals. Thanks to a
307 far greater number of patients and longer follow-up time, we showed here a more extensive
308 increase of the proportion of integrated forms than in this previous work. With techniques
309 allowing the comparison between the levels of total HIV DNA and integrated HIV DNA, we
310 show the benefit of studying the percentage of integrated forms, their heterogeneity between
311 patients and their impact on the outcome of untreated HIV infection. Although several PHI
312 patients had undetectable integrated HIV DNA levels, the technique used here has a low
313 quantification threshold and we are confident these results reflect truly low levels of
314 integrated forms. The longer follow-up time also allowed us to highlight the progressive
315 increase of total HIV DNA, which was not significant in previous shorter studies.

316

317 Taking all these results into account, we propose a model for the evolution of HIV DNA
318 forms in blood during the natural history of HIV infection (Figure 4a). During PHI, the labile
319 linear and episomal HIV DNA forms are particularly abundant and mostly constitute evidence
320 of active replication in recently infected activated cells¹⁷. This observation might be
321 explained by the simultaneous cytokine storm and high immune activation detected at this
322 stage. Later during the first year following infection, while the infection is evolving towards a
323 steady state, integrated HIV DNA becomes the major component of the total HIV DNA in
324 most patients. Both HIV DNA markers continue to increase over the subsequent years of
325 follow-up (Figure 4a). The expansion of HIV reservoirs can be linked to several factors: the
326 infection of new cells due to continuous viral replication, the persistence of long-lived
327 infected cells, and the proliferation of infected cells³³⁻³⁵. The last two factors might explain

328 the increasing contribution of integrated HIV DNA forms, which are transmitted from a
329 mother cell to all daughter cells, while unintegrated forms are diluted during cell division.

330 At the AIDS stage, integrated forms represented the vast majority of HIV DNA in almost all
331 patients, which demonstrates that during this profound immunodeficiency, the presence of
332 stable reservoirs is definitely unaffected by cART.

333

334 The models in Figure 4, depicting the evolution of the different viral forms over time, propose
335 an explanation for why very early treatment is highly beneficial. Such early treatment has
336 been proven to be more effective in reducing total HIV DNA levels and restoring immune
337 functions ^{11,12,36,37}. The rapid and steep initial decline of total HIV DNA (Figure 4b) could be
338 explained mainly by the elimination of the labile unintegrated forms. Early cART initiation
339 can also limit the establishment of persistent integrated HIV DNA forms and prevent the
340 progressive increase of blood reservoirs observed in the natural history of the infection.

341 Moreover, previous studies have shown that HIV-infected blood cells during PHI are mostly
342 short-lived T-cells ³⁸, which is an additional factor contributing to reservoir instability during
343 this stage. Treating HIV-infected individuals as soon as the PHI stage allows partial
344 preservation of the most long-lived memory T-cells from infection and the maintenance of a
345 predominant contribution of the short-lived ones ³⁶⁻⁴⁰. The slower but continued decrease in
346 total HIV DNA at later times following cART initiation might be explained by the death of
347 these short-lived infected cells ^{11,12}. In contrast, in patients treated during the chronic stage
348 (Figure 4c), total HIV DNA decreases only shortly after cART initiation, due to the
349 elimination of infected activated cells ⁴¹. Integrated HIV DNA, which is highly predominant
350 in latently infected cells, persists at high levels thereafter. Long-lived highly proliferative
351 central-memory T-cells have been shown to be the main contributors to HIV blood reservoirs
352 during the chronic stage and in patients treated since the chronic stage, contributing to more

353 stable reservoirs^{35,40}. Taken together, these results suggest that during PHI, the existing
354 reservoirs, which are mainly composed of short-lived cells infected with unstable viral forms,
355 are easily eliminated through the early initiation of cART. In contrast, in the chronic phase,
356 the blood reservoirs are mainly composed of stable proviruses in long-lived quiescent cells
357 and are unaffected by cART. This could explain the greater impact of treatment when
358 initiated during PHI compared to the chronic phase, which is an additional reason for
359 recommending the treatment of all HIV-infected individuals, including the initiation of
360 treatment as soon as possible in those diagnosed during PHI.

361

362 We acknowledge that in the present study we quantified total and integrated HIV DNA on
363 PBMC and not CD4+ T cells. Nevertheless, in a previous study on patients from the same
364 ANRS-SEROCO cohort, we showed a high correlation of total HIV DNA levels expressed
365 either by copies/10⁶ PBMC, or copies/10⁶ CD4+ T-cells or copies/mL of whole blood⁴². The
366 predictive value level on disease progression was similar whatever the expression of the total
367 HIV DNA load. Besides, we only investigated blood reservoirs. Despite the continuous
368 circulation of lymphocytes between the blood and tissues, the dynamics of integrated HIV
369 DNA and total HIV DNA in infected CD4+ T-cell subsets and their relative contributions
370 could differ in lymphoid tissues because of different inflammation and cellular activation
371 levels, which should therefore also be explored.

372

373

374 In conclusion, we used two large cohorts of untreated HIV-infected individuals to describe the
375 evolution of total and integrated HIV DNA loads and their association with disease evolution.
376 We showed that integrated forms constitute a minority of the HIV DNA present during PHI
377 and progressively become predominant over time. However, patients with high levels of

378 integrated HIV DNA shortly after infection are at a higher risk of rapid progression towards
379 AIDS, indicating that the rapidity of progression is determined soon after infection. These
380 results highlight that PHI is a key period, and they contribute to the understanding of why
381 cART initiation during this time is most effective in decreasing the HIV DNA burden, thereby
382 protecting immune cells from infection. Concomitant analysis of total HIV DNA and
383 integrated HIV DNA loads contributes to the understanding of HIV persistence and
384 pathogenesis. Studying both markers may help to identify HIV-infected individuals who will
385 be the best candidates for new therapeutic strategies targeting HIV, including cure and/or
386 remission strategies.

387

388

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395

396 **Declaration of interests**

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400

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405 interpretation, writing the report, or decision to submit for publication.

406

407 **Authors' contributions**

408 VAF, CR, LM, and FB designed the study. PT, TL, FB, CR, LM, and VAF interpreted the
409 results. PT and TL produced the virological data. FB and AE gathered the data from the
410 cohorts. CG, MB, and JPV included patients and collected the clinical data. PT, TL, FB, CR,
411 LM, and VAF analysed the data. PT, TL, FB, OD, JPV, CG, CR, LM, and VAF participated
412 in drafting and/or revising the manuscript. VAF was responsible for virological analyses. LM

413 was responsible for statistical analyses and models. AM, MG, and OD provided technical
414 support. VAF supervised the study. All authors critically reviewed and approved the
415 manuscript. PT and TL, as well as LM and VAF, contributed equally to this work.

416

417 **Note**

418 Abstract: 262 words

419 Body of the text: 3934 words

420

421 **References**

422

- 423 1. Churchill MJ, Deeks SG, Margolis DM, Siliciano RF, Swanstrom R. HIV reservoirs:
424 what, where and how to target them. *Nat Rev Microbiol* 2016; **14**(1): 55-60.
- 425 2. Murray JM, Zaunders JJ, McBride KL, et al. HIV DNA subspecies persist in both
426 activated and resting memory CD4+ T cells during antiretroviral therapy. *J Virol* 2014; **88**(6):
427 3516-26.
- 428 3. Avettand-Fenoel V, Chaix ML, Blanche S, et al. LTR real-time PCR for HIV-1 DNA
429 quantitation in blood cells for early diagnosis in infants born to seropositive mothers treated in
430 HAART area (ANRS CO 01). *J Med Virol* 2009; **81**(2): 217-23.
- 431 4. Bruner KM, Murray AJ, Pollack RA, et al. Defective proviruses rapidly accumulate
432 during acute HIV-1 infection. *Nat Med* 2016; **22**(9): 1043-9.
- 433 5. Ho YC, Shan L, Hosmane NN, et al. Replication-competent noninduced proviruses in
434 the latent reservoir increase barrier to HIV-1 cure. *Cell* 2013; **155**(3): 540-51.
- 435 6. Plantin J, Massanella M, Chomont N. Inducible HIV RNA transcription assays to
436 measure HIV persistence: pros and cons of a compromise. *Retrovirology* 2018; **15**(1): 9.
- 437 7. Descours B, Avettand-Fenoel V, Blanc C, et al. Immune responses driven by
438 protective human leukocyte antigen alleles from long-term nonprogressors are associated with
439 low HIV reservoir in central memory CD4 T cells. *Clin Infect Dis* 2012; **54**(10): 1495-503.
- 440 8. Sharkey M, Triques K, Kuritzkes DR, Stevenson M. In vivo evidence for instability of
441 episomal human immunodeficiency virus type 1 cDNA. *J Virol* 2005; **79**(8): 5203-10.
- 442 9. Mexas AM, Graf EH, Pace MJ, et al. Concurrent measures of total and integrated HIV
443 DNA monitor reservoirs and ongoing replication in eradication trials. *AIDS* 2012; **26**(18):
444 2295-306.
- 445 10. Avettand-Fenoel V, Hocqueloux L, Ghosn J, et al. Total HIV-1 DNA, a Marker of
446 Viral Reservoir Dynamics with Clinical Implications. *Clin Microbiol Rev* 2016; **29**(4): 859-80.
- 447 11. Hocqueloux L, Avettand-Fenoel V, Jacquot S, et al. Long-term antiretroviral therapy
448 initiated during primary HIV-1 infection is key to achieving both low HIV reservoirs and
449 normal T cell counts. *J Antimicrob Chemother* 2013; **68**(5): 1169-78.
- 450 12. Laanani M, Ghosn J, Essat A, et al. Impact of the Timing of Initiation of Antiretroviral
451 Therapy During Primary HIV-1 Infection on the Decay of Cell-Associated HIV-DNA. *Clin*
452 *Infect Dis* 2015; **60**(11): 1715-21.
- 453 13. Koelsch KK, Boesecke C, McBride K, et al. Impact of treatment with raltegravir
454 during primary or chronic HIV infection on RNA decay characteristics and the HIV viral
455 reservoir. *AIDS* 2011; **25**(17): 2069-78.

- 456 14. Murray JM, McBride K, Boesecke C, et al. Integrated HIV DNA accumulates prior to
457 treatment while episomal HIV DNA records ongoing transmission afterwards. *AIDS* 2012;
458 **26**(5): 543-50.
- 459 15. Kiselinova M, De Spiegelaere W, Buzon MJ, Malatinkova E, Lichterfeld M,
460 Vandekerckhove L. Integrated and Total HIV-1 DNA Predict Ex Vivo Viral Outgrowth. *PLoS*
461 *Pathog* 2016; **12**(3): e1005472.
- 462 16. Pinzone MR, Graf E, Lynch L, et al. Monitoring Integration over Time Supports a
463 Role for Cytotoxic T Lymphocytes and Ongoing Replication as Determinants of Reservoir
464 Size. *J Virol* 2016; **90**(23): 10436-45.
- 465 17. Zhu W, Jiao Y, Lei R, et al. Rapid turnover of 2-LTR HIV-1 DNA during early stage
466 of highly active antiretroviral therapy. *PLoS One* 2011; **6**(6): e21081.
- 467 18. Ananworanich J, Chomont N, Eller LA, et al. HIV DNA Set Point is Rapidly
468 Established in Acute HIV Infection and Dramatically Reduced by Early ART. *EBioMedicine*
469 2016; **11**: 68-72.
- 470 19. Eriksson S, Graf E, Dahl V, et al. Comparative analysis of measures of viral reservoirs
471 in HIV-1 eradication studies. *PLoS Pathog* 2013; **9**(2): e1003174.
- 472 20. Cortes FH, Passaes CP, Bello G, et al. HIV controllers with different viral load cutoff
473 levels have distinct virologic and immunologic profiles. *J Acquir Immune Defic Syndr* 2015;
474 **68**(4): 377-85.
- 475 21. Vandergeeten C, Fromentin R, Merlini E, et al. Cross-clade Ultrasensitive PCR-based
476 Assays to Measure HIV Persistence in Large Cohort Studies. *J Virol* 2014.
- 477 22. Ghosn J, Deveau C, Chaix ML, et al. Despite being highly diverse, immunovirological
478 status strongly correlates with clinical symptoms during primary HIV-1 infection: a cross-
479 sectional study based on 674 patients enrolled in the ANRS CO 06 PRIMO cohort. *J*
480 *Antimicrob Chemother* 2010; **65**(4): 741-8.
- 481 23. Hubert JB, Burgard M, Dussaix E, et al. Natural history of serum HIV-1 RNA levels
482 in 330 patients with a known date of infection. The SEROCO Study Group. *Aids* 2000; **14**(2):
483 123-31.
- 484 24. Brussel A, Delelis O, Sonigo P. Alu-LTR real-time nested PCR assay for quantifying
485 integrated HIV-1 DNA. *Methods Mol Biol* 2005; **304**: 139-54.
- 486 25. O'Doherty U, Swiggard WJ, Jeyakumar D, McGain D, Malim MH. A sensitive,
487 quantitative assay for human immunodeficiency virus type 1 integration. *J Virol* 2002;
488 **76**(21): 10942-50.
- 489 26. Pinzone MR, Di Rosa M, Cacopardo B, Nunnari G. HIV RNA suppression and
490 immune restoration: can we do better? *Clin Dev Immunol* 2012; **2012**: 515962.
- 491 27. Rouzioux C, Hubert JB, Burgard M, et al. Early Levels of HIV-1 DNA in Peripheral
492 Blood Mononuclear Cells Are Predictive of Disease Progression Independently of HIV-1
493 RNA Levels and CD4+ T Cell Counts. *J Infect Dis* 2005; **192**(1): 46-55. Epub 2005 May 31.

- 494 28. Imamichi H, Dewar RL, Adelsberger JW, et al. Defective HIV-1 proviruses produce
495 novel protein-coding RNA species in HIV-infected patients on combination antiretroviral
496 therapy. *Proc Natl Acad Sci U S A* 2016; **113**(31): 8783-8.
- 497 29. Goujard C, Bonarek M, Meyer L, et al. CD4 cell count and HIV DNA level are
498 independent predictors of disease progression after primary HIV type 1 infection in untreated
499 patients. *Clin Infect Dis* 2006; **42**(5): 709-15.
- 500 30. Graf EH, Mexas AM, Yu JJ, et al. Elite suppressors harbor low levels of integrated
501 HIV DNA and high levels of 2-LTR circular HIV DNA compared to HIV+ patients on and
502 off HAART. *PLoS Pathog* 2011; **7**(2): e1001300.
- 503 31. Pasternak AO, Lukashov VV, Berkhout B. Cell-associated HIV RNA: a dynamic
504 biomarker of viral persistence. *Retrovirology* 2013; **10**: 41.
- 505 32. Ananworanich J, Sacdalan CP, Pinyakorn S, et al. Virological and immunological
506 characteristics of HIV-infected individuals at the earliest stage of infection. *J Virus Erad*
507 2016; **2**: 43-8.
- 508 33. Bacchus-Souffan C FM, Abdel-Mohsen M, Hoh R, Ahn H, Hecht F, Martin J, Deeks
509 S, Liegler T, Hunt M, Hellerstein M, McCune JM. Early initiation of long-term ART may
510 favor interventions to eradicate HIV: analysis of cell turnover and persistent HIV reservoirs
511 in distinct CD4+ T cells in suppressed HIV disease. 9th International AIDS Society
512 Conference. Paris, France; 2017.
- 513 34. Pasternak AO, Jurriaans S, Bakker M, Berkhout B, Lukashov VV. Steady increase in
514 cellular HIV-1 load during the asymptomatic phase of untreated infection despite stable
515 plasma viremia. *AIDS* 2010; **24**(11): 1641-9.
- 516 35. Chomont N, El-Far M, Ancuta P, et al. HIV reservoir size and persistence are driven
517 by T cell survival and homeostatic proliferation. *Nat Med* 2009; **15**(8): 893-900.
- 518 36. Li JZ, Gandhi RT. The sooner, the better: more evidence that early antiretroviral
519 therapy lowers viral reservoirs in HIV-infected infants. *J Infect Dis* 2014; **210**(10): 1519-22.
- 520 37. Ananworanich J, Vandergeeten C, Chomchey N, et al. Early ART Intervention
521 Restricts the Seeding of the HIV Reservoir in Long-lived Central Memory CD4 T Cells.
522 *CROI* 2013; **Abst. 47**.
- 523 38. Cheret A, Bacchus-Souffan C, Avettand-Fenoel V, et al. Combined ART started
524 during acute HIV infection protects central memory CD4+ T cells and can induce remission. *J*
525 *Antimicrob Chemother* 2015; **70**(7): 2108-20.
- 526 39. Bacchus C, Cheret A, Avettand-Fenoel V, et al. A single HIV-1 cluster and a skewed
527 immune homeostasis drive the early spread of HIV among resting CD4+ cell subsets within
528 one month post-infection. *PLoS One* 2013; **8**(5): e64219.
- 529 40. Gantner P, Barnig C, Partisani M, et al. Distribution and reduction magnitude of HIV-
530 DNA burden in CD4+ T cell subsets depend on art initiation timing. *AIDS* 2018; **32**(7): 921-6.
- 531 41. Murray JM, Zaunders J, Emery S, et al. HIV dynamics linked to memory CD4+ T cell
532 homeostasis. *PLoS One* 2017; **12**(10): e0186101.

- 533 42. Avettand-Fenoel V, Boufassa F, Galimand J, Meyer L, Rouzioux C, Group ASCS.
534 HIV-1 DNA for the measurement of the HIV reservoir is predictive of disease progression in
535 seroconverters whatever the mode of result expression is. *J Clin Virol* 2008; **42**(4): 399-404.
- 536 43. Cardozo EF, Andrade A, Mellors JW, Kuritzkes DR, Perelson AS, Ribeiro RM.
537 Treatment with integrase inhibitor suggests a new interpretation of HIV RNA decay curves
538 that reveals a subset of cells with slow integration. *PLoS Pathog* 2017; **13**(7): e1006478.
- 539 44. Kotton CN, Kumar D, Caliendo AM, et al. International consensus guidelines on the
540 management of cytomegalovirus in solid organ transplantation. *Transplantation* 2010; **89**(7):
541 779-95.
542
- 543

544 **Tables and figures**

545

546 **Table 1. Baseline demographic and clinical characteristics of the patients.**

Characteristics	PHI cohort ^a	Progression cohort ^a	<i>p</i> value ^b	Among the progression cohort		
				Rapid progressors	Slower progressors	<i>p</i> value ^b
n	74	97		34	63	
Male, n (%)	69 (93.2%)	79 (81.4%)	0.040	29 (36.7%)	50 (63.3%)	0.589
Caucasians, n (%)	61 (82.4%)	95 (97.9%)	<0.001	34 (100.0%)	61 (96.8%)	0.540
Median age at inclusion (IQR), years	36 (29-45)	30 (26-36)	<0.001	31 (26-37)	30 (26-36)	0.530
Sexual route of infection, n (%)	72 (97.3%)	88 (90.7%)	0.117	32 (94.1%)	56 (88.9%)	0.487
Median time between seroconversion and first sample (IQR), months	1.0 (0.8-1.3)	6.2 (4.2-8.5)	<0.001	7.1 (4.4-9.5)	6.0 (4.2-8.3)	0.415
Median time of follow-up (IQR), years	na	na	na	4.5 (3.5-6.0)	5.8 (4.6-6.3)	0.008
Median baseline CD4+ cell count (IQR), cells/mm ³	449 (317-613)	531 (390-666)	0.036	481 (343-608)	544 (429-706)	0.124
Median baseline CD8+ cell count (IQR), cells/mm ³	761 (505-1253)	846 (639-1157)	0.177	927 (632-1157)	832 (650-1144)	0.972
Median baseline CD4+/CD8+ cell ratio	0.60 (0.37-0.91)	0.57 (0.44-0.87)	0.999	0.52 (0.41-0.77)	0.65 (0.45-0.88)	0.308
Median plasma HIV RNA load (IQR), log copies/mL	6.20 (5.30-6.98)	4.16 (3.59-4.52)	<0.001	4.37 (3.96-4.67)	3.98 (3.47-4.39)	0.002
Median total HIV DNA load (IQR), log copies/10 ⁶ PBMCs	3.59 (3.29-4.03)	3.10 (2.73-3.46)	<0.001	3.22 (3.01-3.59)	2.96 (2.63-3.38)	0.008
Median integrated HIV DNA load (IQR), log copies/10 ⁶ PBMCs	2.15 (0.95-3.16)	2.90 (2.17-3.40)	0.003	3.32 (2.97-3.66)	2.53 (1.52-3.07)	<0.001

547 ^a PHI refers to patients in the primary HIV infection stage (<3 months since the estimated infection date, ANRS-PRIMO
548 cohort), while patients from the progression cohort were in their first year of infection (ANRS-SEROCO cohort). Rapid and
549 slower progressors groups are defined according to their subsequent progression to clinical AIDS.

550 ^b obtained from Fisher's exact or Wilcoxon rank-sum tests.

551 na: non-applicable

552

553 **Table 2. Factors associated with the evolution towards clinical AIDS according to**
554 **baseline levels of total HIV DNA and integrated HIV DNA in univariate and**
555 **multivariate analyses.**

556

		Univariate analysis			Multivariate analysis including total HIV DNA			Multivariate analysis including integrated HIV DNA		
		RR	95% CI	<i>p</i> value	aRR	95% CI	<i>p</i> value	aRR	95% CI	<i>p</i> value
Sex	male	1			1			1		
	female	0.50	[0.19 – 1.32]	0.161	1.00	[0.34 – 2.99]	0.995	1.09	[0.38 – 3.14]	0.870
Age at inclusion ^a		1.02	[0.98 - 1.06]	0.249	1.01	[0.97 – 1.05]	0.737	0.70	[0.97 – 1.05]	0.699
log ₁₀ HIV RNA (copies/mL) ^b		2.48	[1.45 – 4.23]	0.001	2.02	[1.02 – 4.00]	0.044	1.42	[0.74 – 2.73]	0.290
log ₁₀ total HIV DNA (copies/10 ⁶ PBMCs) ^b		2.55	[1.24 – 5.22]	0.011	1.47	[0.61 – 3.54]	0.392			
log ₁₀ integrated HIV DNA (copies/10 ⁶ PBMCs) ^b		3.12	[1.78 – 5.46]	0.000				2.63	[1.41 – 4.91]	0.002

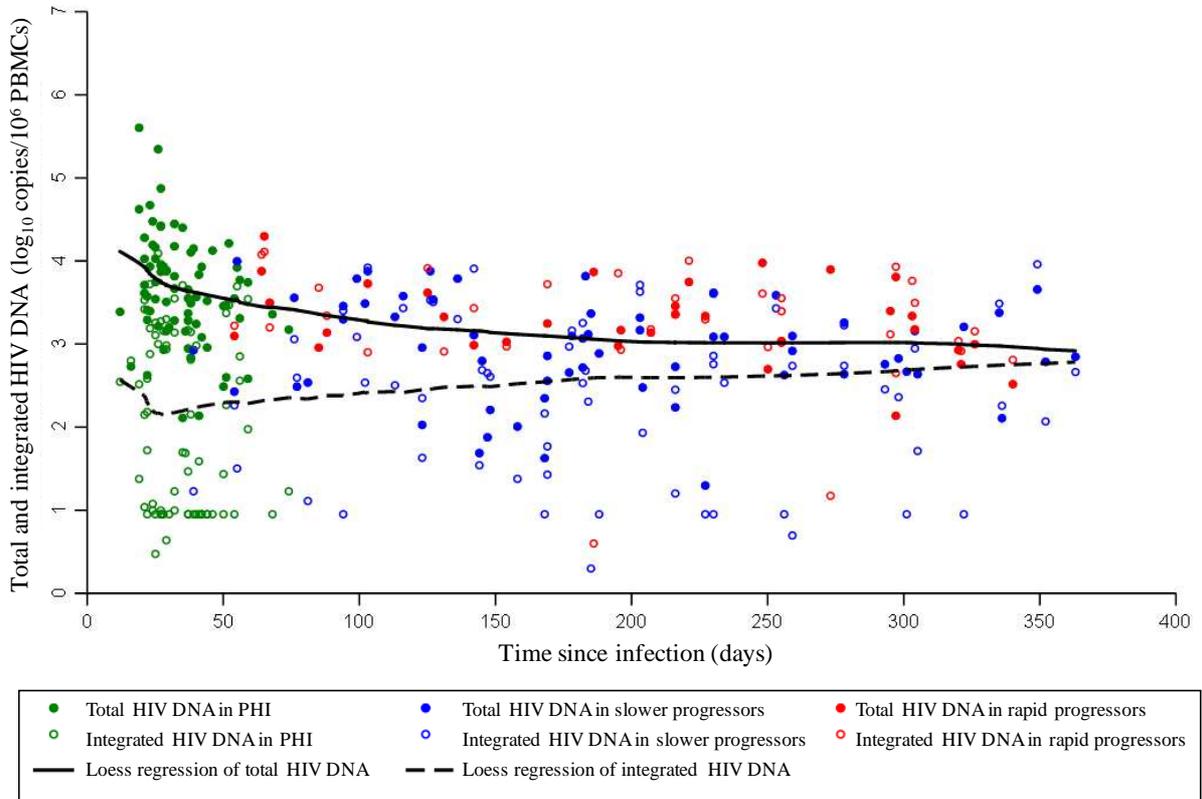
^a for a one-year positive difference; ^b for a one-log positive difference

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558

559 **Figure 1. Total HIV DNA and integrated HIV DNA loads in the first year following**
 560 **infection**

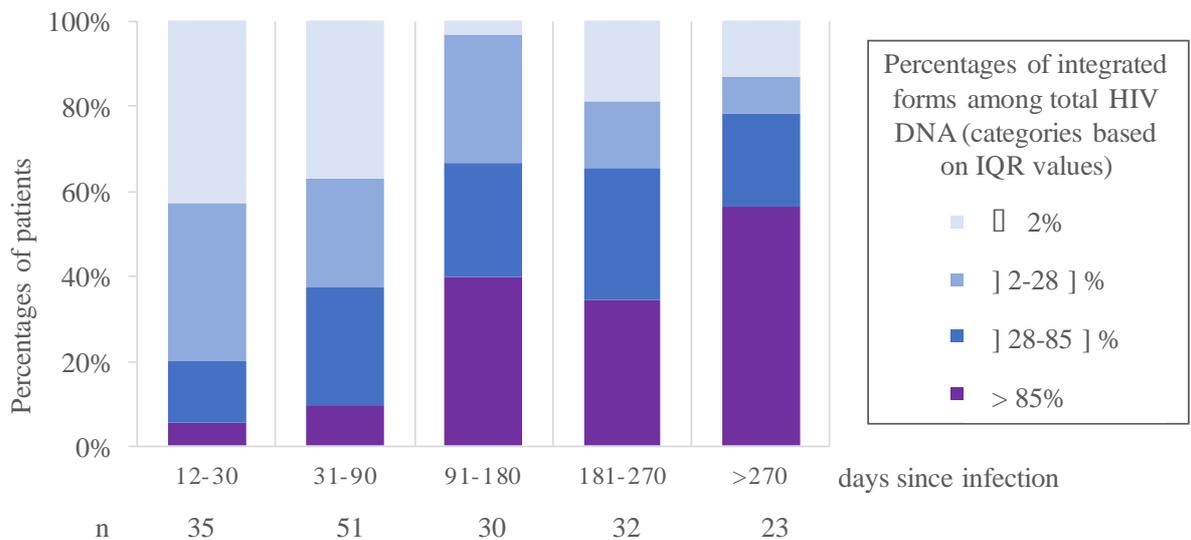
561 **a.**



562

563

564 **b.**



565

566

567 (a) Distribution with Loess regression of total HIV DNA (filled circles and solid line) and integrated HIV DNA
568 (open circles and dotted line) according to the estimated time since infection (171 patients, one time point per
569 patient at their inclusion in the ANRS-PRIMO cohort (PHI) or the ANRS-SEROCO cohort (progression cohort,
570 divided into rapid and slower progressors)).

571 (b) Repartition of integrated among total HIV DNA percentages according to the time since infection. These
572 percentages were divided into four categories defined by the quartile values obtained for the 171 patients (light
573 blue to purple, with Q1=2%, Q2=28%, Q3=85%). Patients were classified into five groups defined by the time
574 since infection (in days). The bar chart represents the percentage of patients belonging to each category of
575 integrated/total HIV DNA percentage.

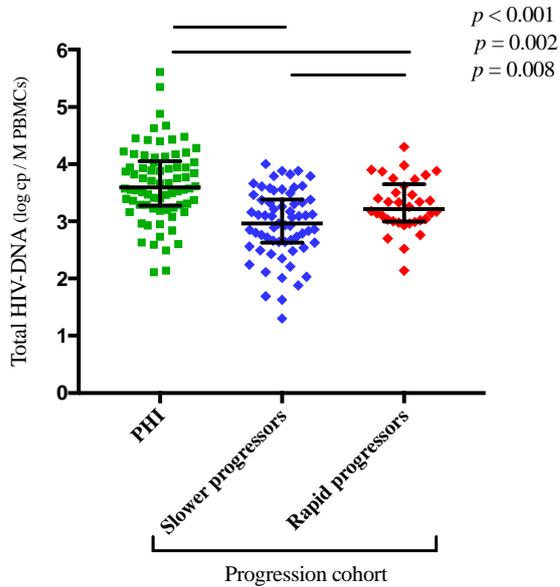
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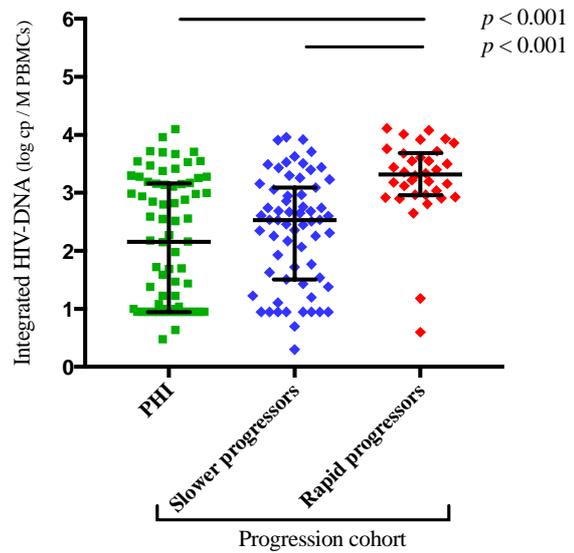
578 **Figure 2. Baseline HIV DNA levels during primary infection and recent seroconversion,**
579 **according to further progression to clinical AIDS.**

580

581 **a.**



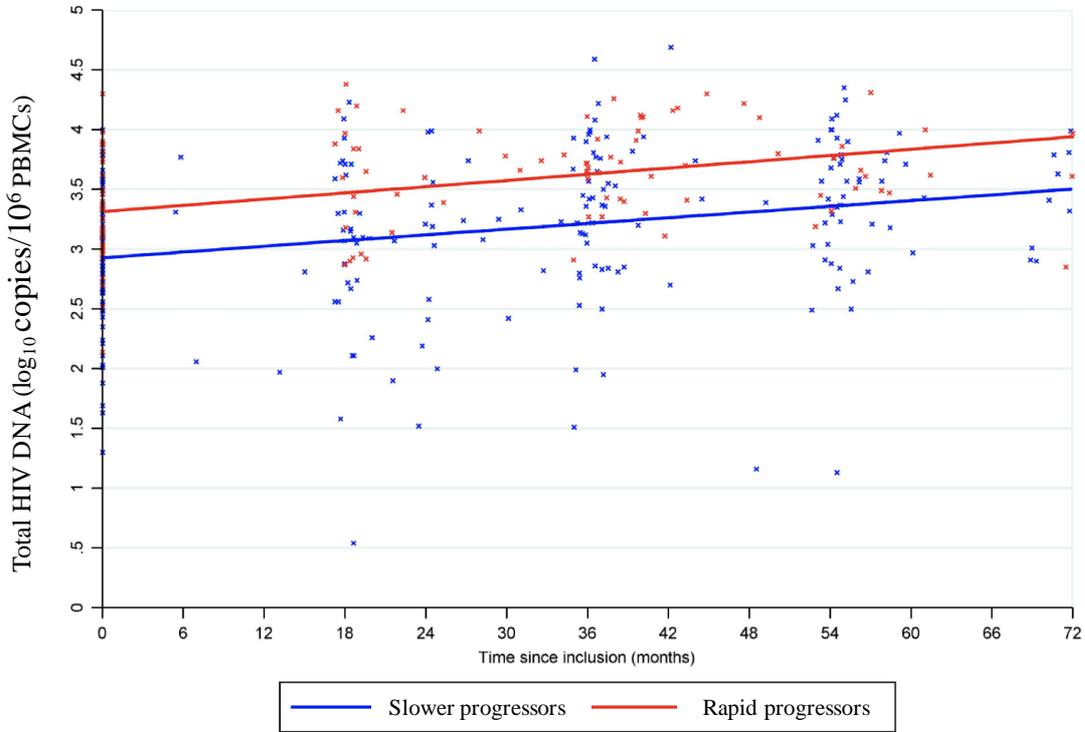
b.



584 Scatter dot plots display (a) total HIV DNA and (b) integrated HIV DNA loads for patients in the primary
585 infection phase (PHI, ANRS-PRIMO cohort, <3 months since the estimated time of infection) and for the rapid
586 progressors and slower progressors groups among the progression cohort (ANRS-SEROCO cohort, <1 year
587 since infection). The bars represent median and interquartile range values.

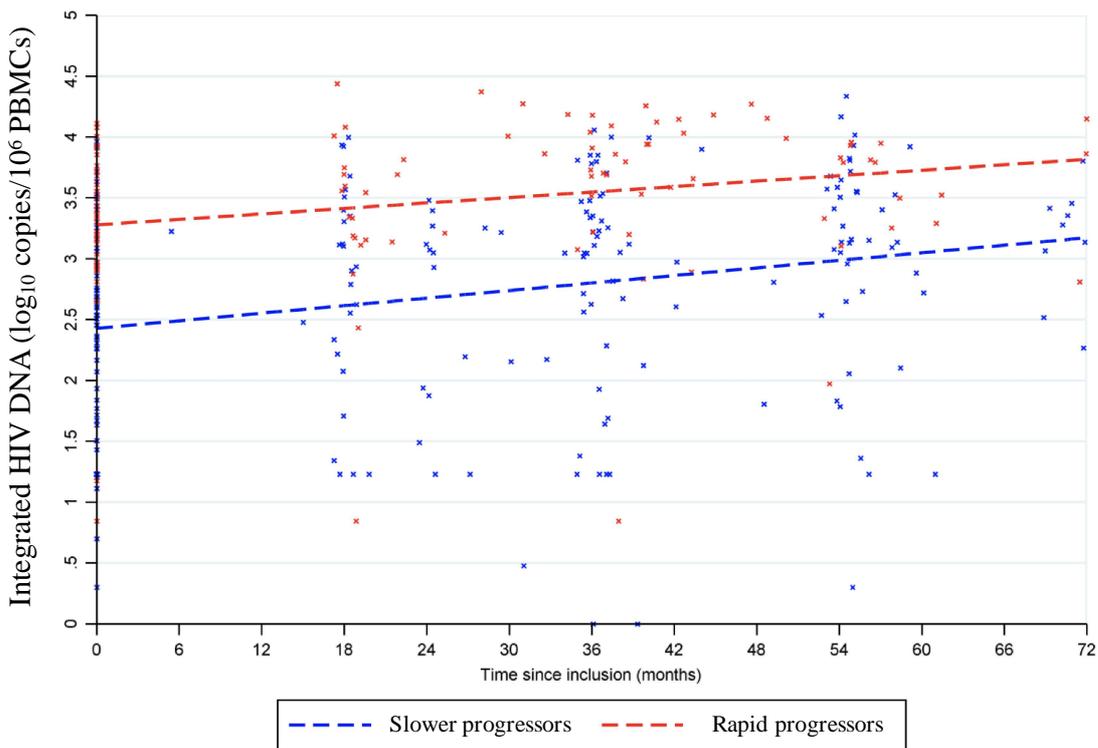
590 **Figure 3. Increase in total HIV DNA and integrated HIV DNA over six years of**
591 **untreated infection**

592 **a.**



593

594 **b.**



595

596

597 (a) Mixed-effect linear models (MELMs) describing the evolution of total HIV DNA over six years for rapid
598 progressors (n=34, 111 samples) and slower progressors (n=63, 229 samples).

599 (b) MELMs describing the evolution of integrated HIV DNA over six years for rapid progressors (107 samples)
600 and slower progressors (207 samples).

601 Samples obtained after six years of follow-up were not considered for inclusion in the MELMs to have enough
602 results at each time point.

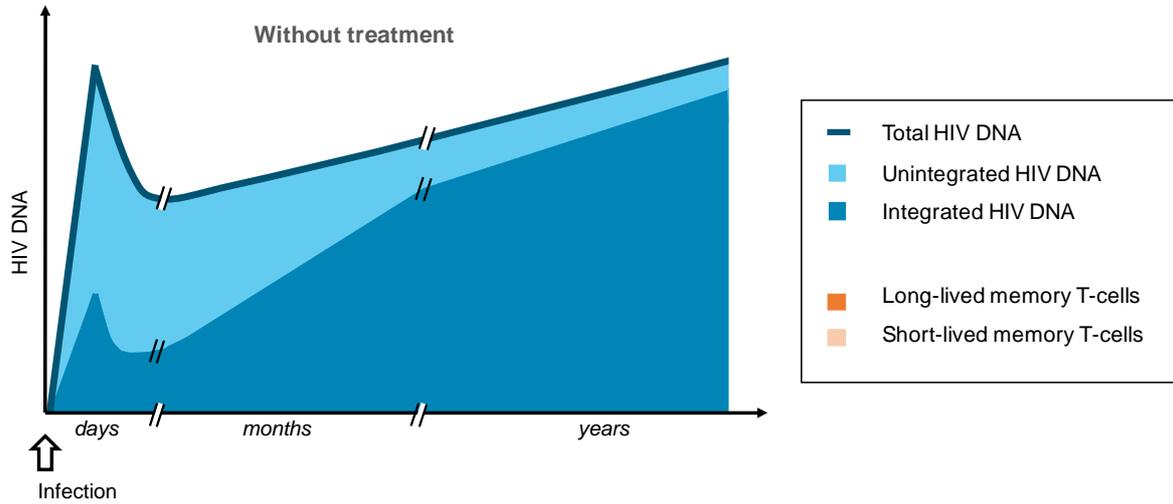
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605 **Figure 4. Proposed models for the dynamics of the different HIV DNA forms in blood**
 606 **reservoirs**

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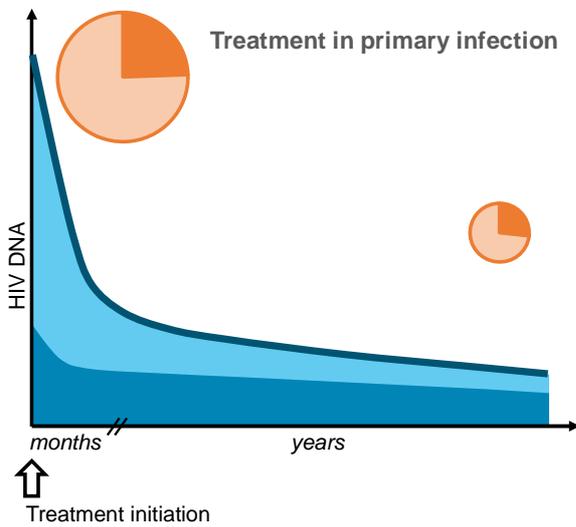
608 **a.**



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611 **b.**

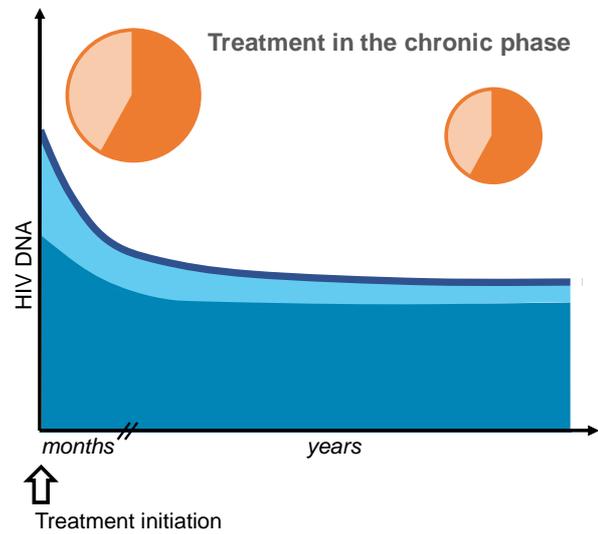


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613

614

c.



615 HIV DNA loads are represented in \log_{10} copies/ 10^6 PBMC. Note the breaks in the time axes.

616 (a) Model for HIV DNA dynamics during the natural history of infection. During early PHI, total HIV DNA
617 levels rise rapidly to reach a peak. Integrated HIV DNA shows a concomitant but much lower peak; the majority
618 of HIV DNA is composed of unintegrated forms at that time¹⁸. Both total HIV DNA and integrated HIV DNA
619 display a slight decrease thereafter, before reaching a more stable state. During chronic infection, both HIV
620 blood biomarkers progressively increase, and the proportion of integrated forms increases.

621 (b, c) Models of HIV DNA dynamics under treatment, depending on the timing of cART initiation. Efficient
622 treatment initiation stops or significantly decreases viral replication and the infection of new cells⁴³.
623 Unintegrated HIV DNA forms, resulting from viral replication, are eliminated because of their lability or cell
624 death, or they are diluted during cell division, without new production as a result of cART. In contrast, stable
625 integrated HIV DNA can persist for a longer time. Thus, cART initiation at PHI (b), when the integrated HIV
626 DNA level is low, induces a more pronounced and extended decrease of total HIV DNA than cART initiation
627 during the chronic stage, when the predominance of stable integrated forms is already higher (c)^{11,12}. Efficient
628 treatment initiation stops the evolution of CD4+ T-cell subsets contributions to HIV reservoirs; they remain
629 mainly composed of short-lived cells when cART is initiated during PHI^{38,40}, while long-lived cells are the
630 major contributor to HIV DNA when cART is initiated during chronic infection^{35,40}. This difference might
631 explain the continued decrease in total HIV DNA after several years of treatment when cART is initiated early.

632

633 **Supplementary material list**

634

635 **Supplementary Table 1.** Lists of the SEROCO/HEMOCO Study Group members and
636 PRIMO Study Group members.

637 **Supplementary Text 1.** Integrated HIV DNA quantification method.

638 **Supplementary Figure 1.** Plasma HIV RNA load in the first year following infection.

639 **Supplementary Figure 2.** Correlation between total HIV DNA and integrated HIV DNA
640 loads for the 171 patients at inclusion.

641 **Supplementary Figure 3.** Correlation between HIV RNA and total HIV DNA loads for the
642 171 patients at inclusion.

643 **Supplementary Figure 4.** Correlation between HIV RNA and integrated HIV DNA loads for
644 the 171 patients at inclusion.

645 **Supplementary Figure 5.** Correlation between HIV RNA and unintegrated HIV DNA loads
646 for the 171 patients at inclusion.

647 **Supplementary Figure 6.** Dynamics of CD4⁺ T-cell counts in the natural history of HIV
648 infection since recent seroconversion.

649 **Supplementary Table 2.** STROBE checklist.

650