



Greater diversity of HIV DNA variants in the rectum compared to variants in the blood in patients without HAART

Véronique Avettand-Fènoël, Laurent Hocqueloux, Michaela Muller-Trutwin, Thierry Prazuck, Adeline Mélard, Marie-Laure Chaix, Eric Agoute, Christophe Michau, Christine Rouzioux

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**Greater diversity of HIV DNA variants in the rectum
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Keywords:	HIV reservoir, gut-associated lymphoid tissue, rectum, phylogeny, diversity

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Table 1. Virologic, immunologic and clinical characteristics of patients

		Zenith CD4			CD4+ cell count			HIV DNA, Log		
		Duration	HIV	T				cp/million cells		
		Stage of	of	RNA	cell	Treatment	Blood	Rectum	Plasma	
Pt	Sex	infection	infection,	Log ₁₀	nadir	(tritherapy)	/mm ³	/million	HIV RNA,	
		months	copies	cells				cells	log cp/mL	PBMCs
			/mL	/mm ³						rectum
A	M	PI	3 *	5.8	267	naïve	322	NA	5.8	3.3
B	M	PI	1 *	5.2	729	naïve	729	NA	5.1	3.6
			3.5 *	5.2	729	for 2.5 months	850	NA	<1.7	3.2
C	M	CI	22	5.7	261	naïve	469	20770	4.6	3.3
			37	5.7	251	naïve	251	NA	5.7	3.6
D	M	CI	117	4.9	377	no	425	7310	4.3	3.7
E	M	CI	122	4.4	105	for 4 years	270	NA	<1.7	3.5
F	F	CI	64	5.3	222	for 3 years	426	32890	<1.7	3.5
G	F	AIDS	120	6.0	15	no	15	5600	6.0	3.8

M: male, F: female, PI: primary infection, CI: chronic infection, PBMCs: peripheral blood mononuclear cells, cp: copy

no: no treatment

NA: not available

* after onset of acute retroviral illness

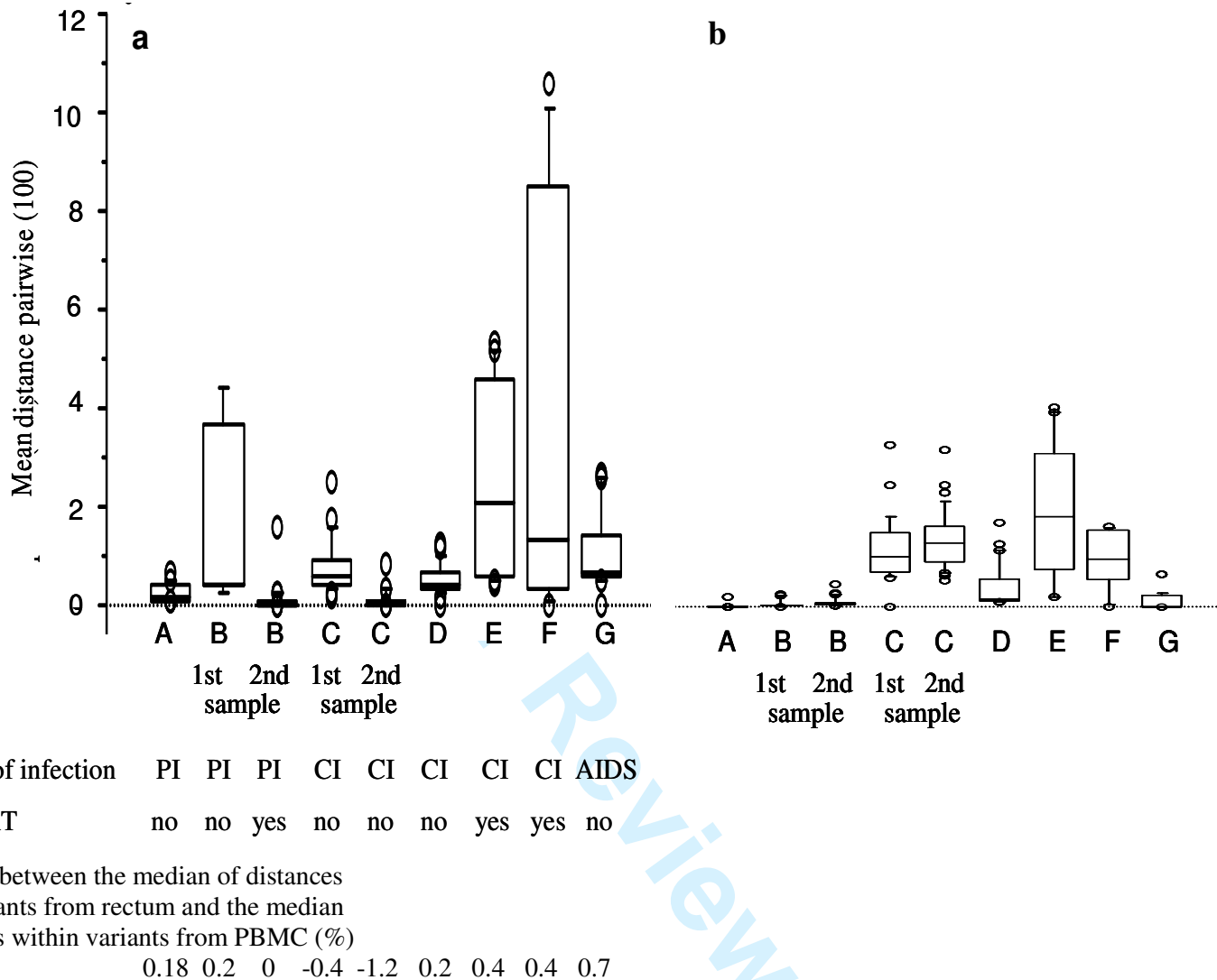


Fig. 1.

Distribution of pairwise distances among rectal HIV-DNA variants (Fig. 1a) and PBMC HIV-DNA variants (Fig. 1b). These boxplots represent the averages of comparisons of pairwise genetic distances from each clone derived from rectal HIV-DNA (Fig. 1a) or from PBMC HIV-DNA (Fig. 1b) to every other clone from the same tissue/fluid. Distances were computed by using the Kimura model and are displayed as 100 times the raw distance score. The horizontal lines show the median and the boxes comprise the first to third quartiles of calculated average genetic distances. A, B, C, D, E, F and G

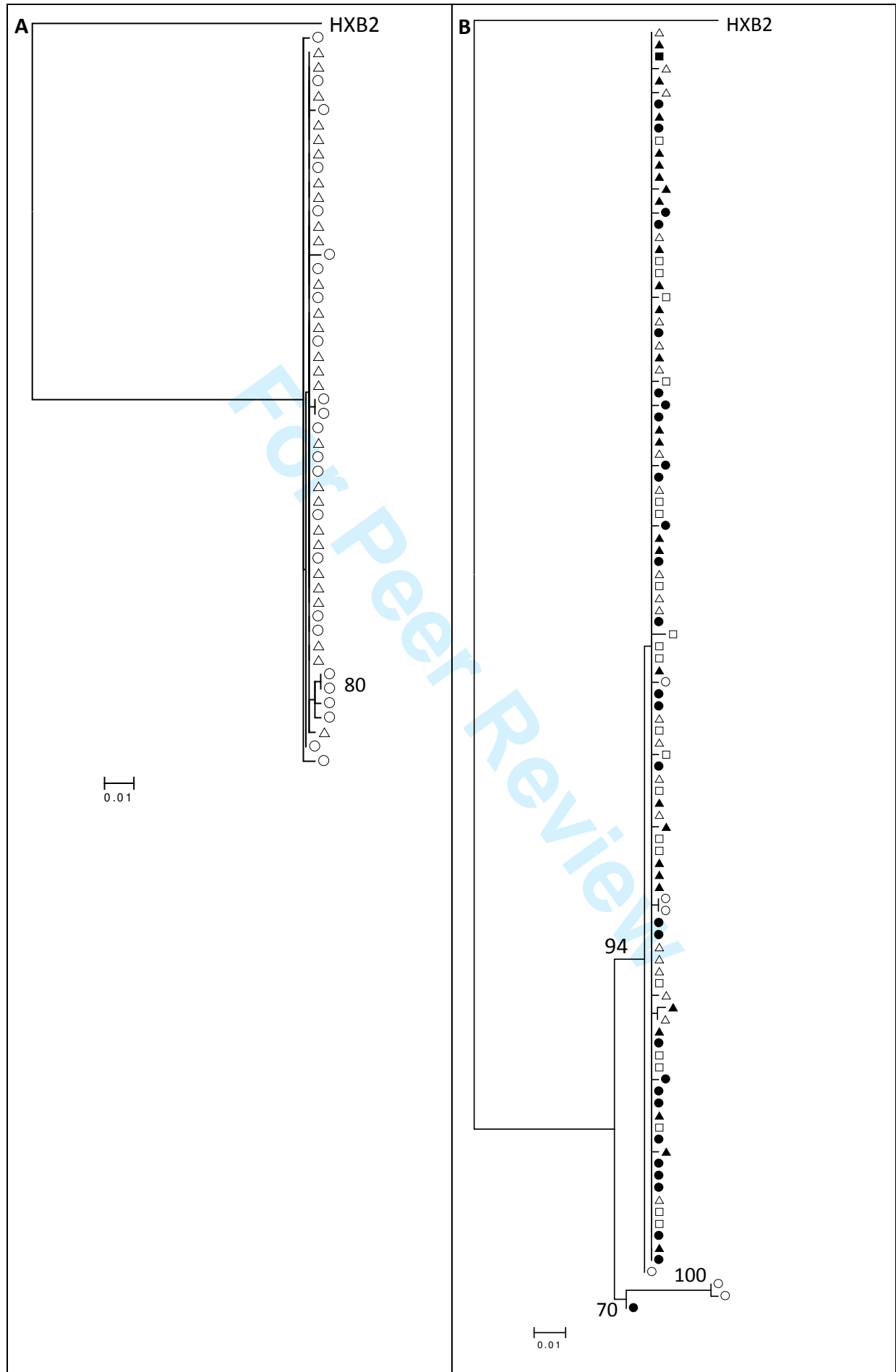
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concern viral clones from patient A, B, C, D, E, F and G, respectively. Significant differences between pairwise rectum HIV-DNA distances and pairwise PBMC HIV-DNA distances were found for each patient ($p<0.01$, Mann-Whitney test), except for patients under treatment (B: 2nd set of samples, E and F).

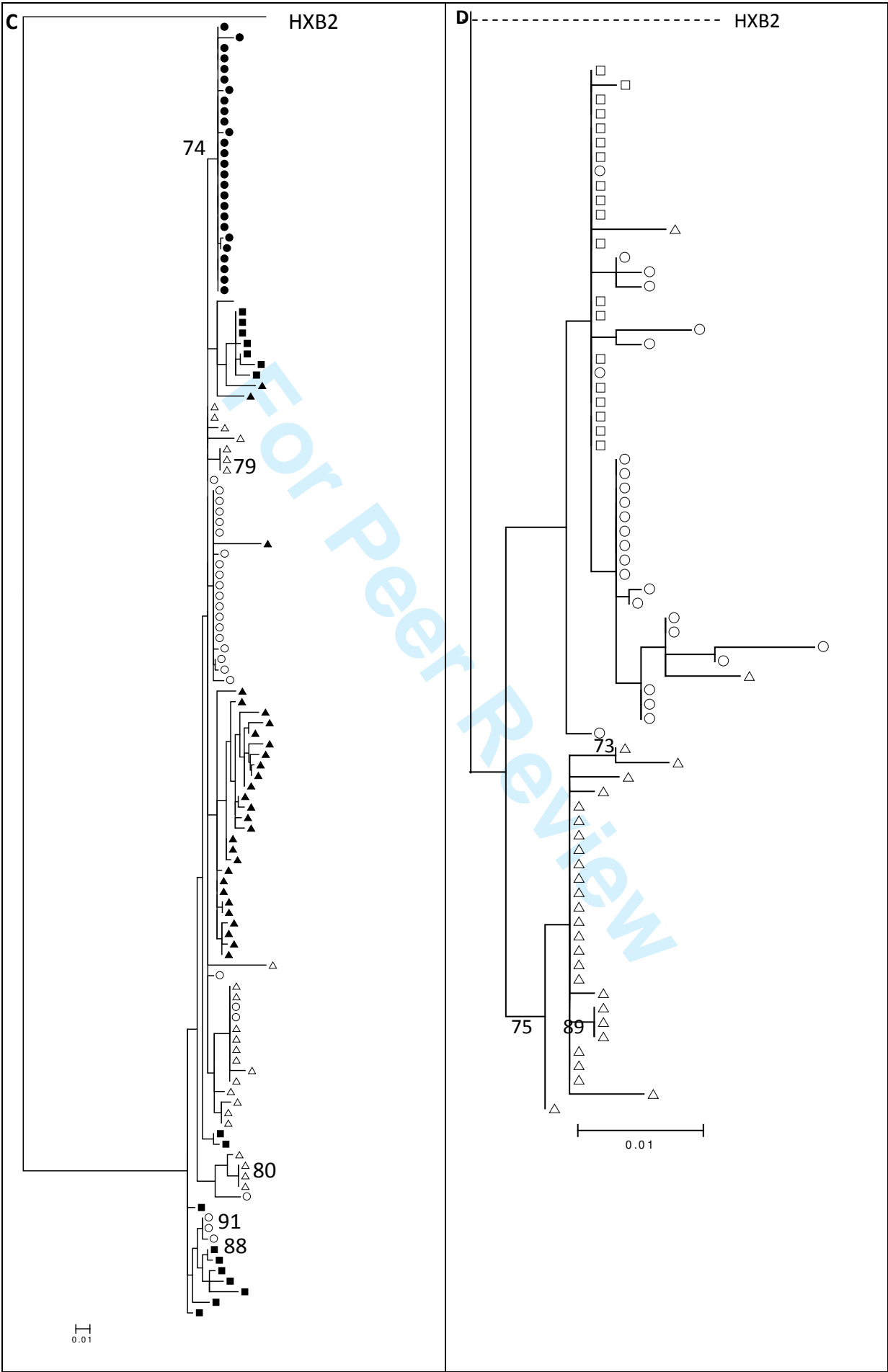
PI: primary infection, CI: chronic infection

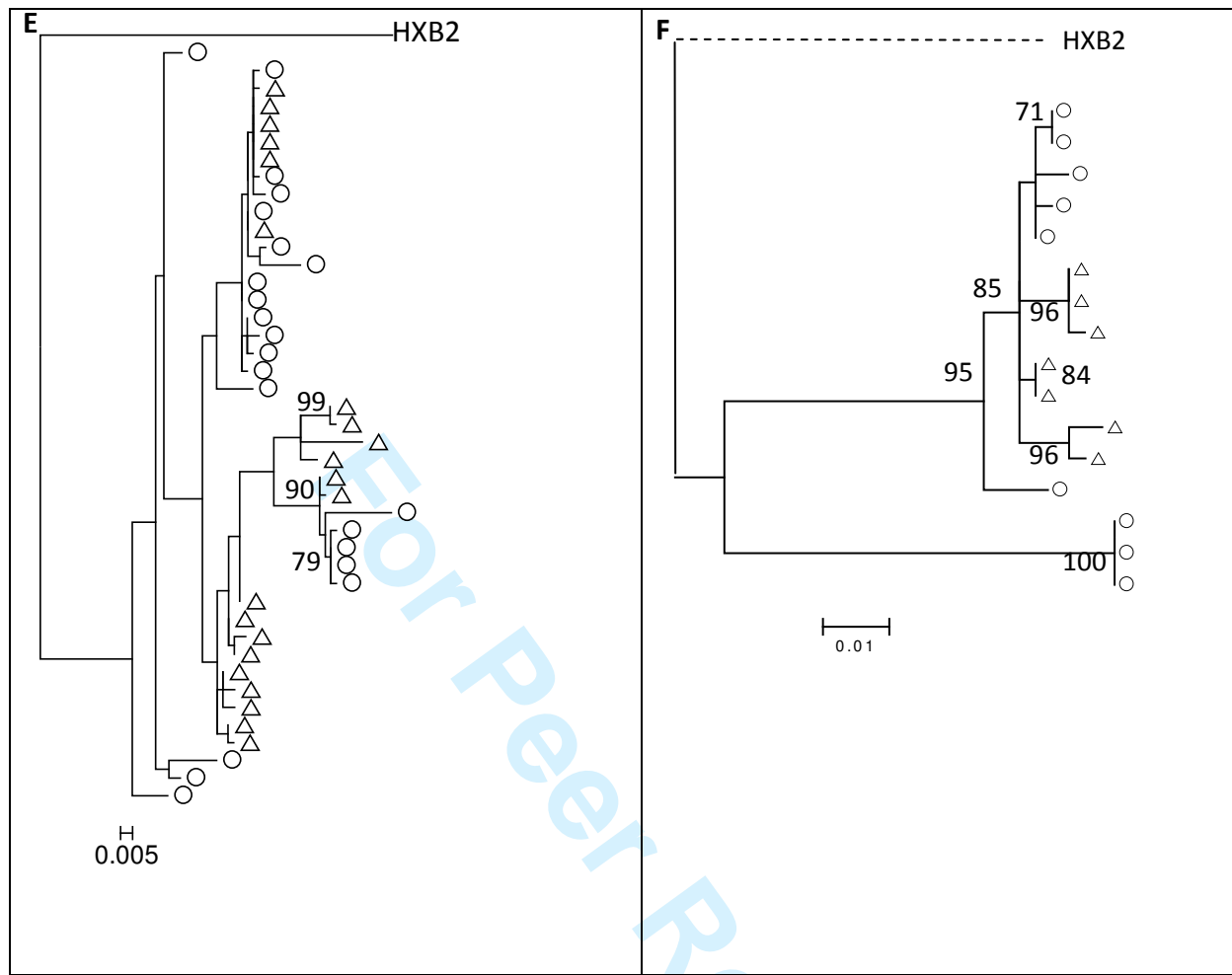
Patients A, B (1st sample), C are naïve to HAART; Patients D and G had received HAART but, at the time of sample collection, were not receiving treatment.

For Peer Review



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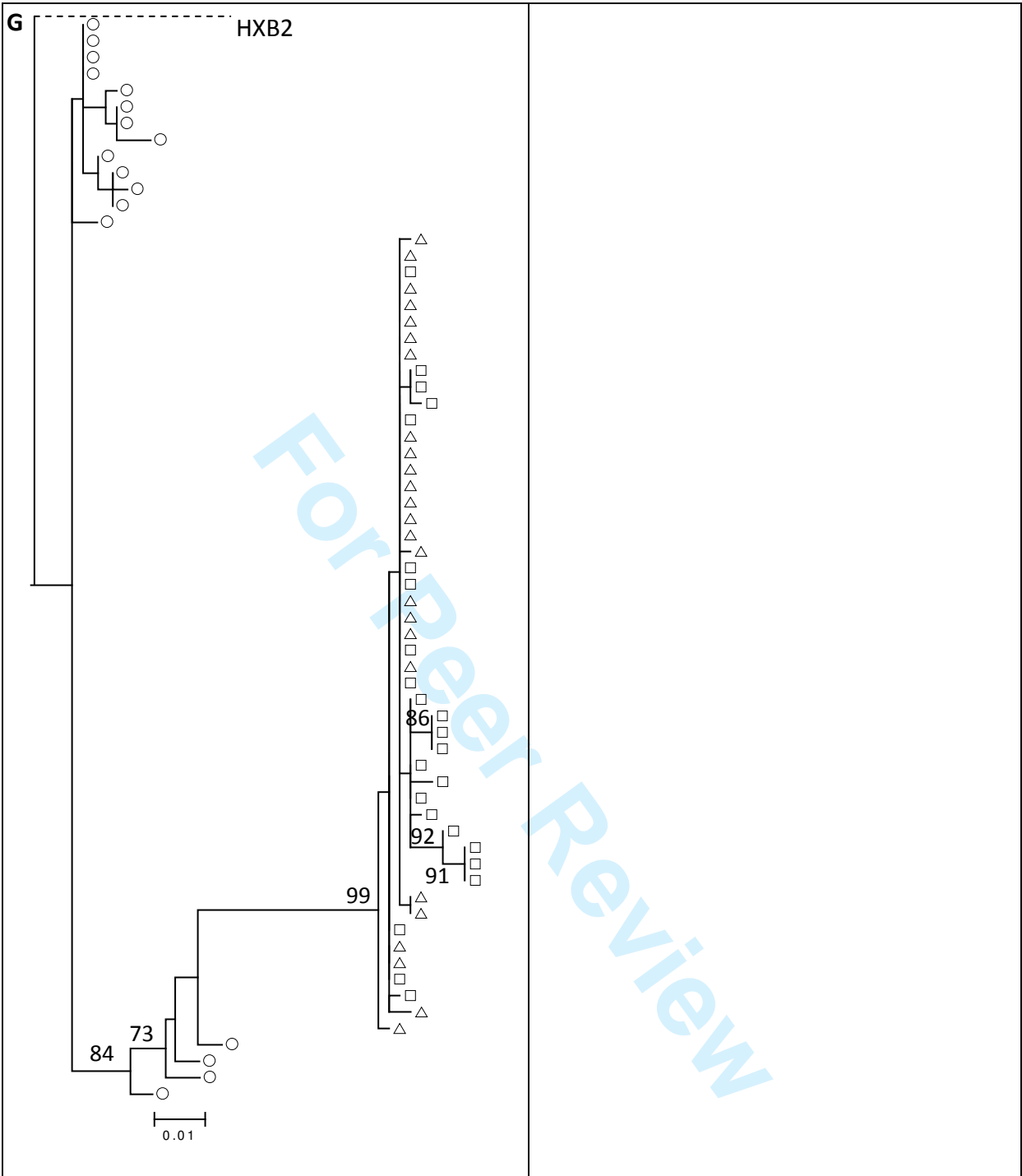


Fig. 2. Phylogenetic relationship of viral clones derived from the blood and rectum (maximum-likelihood trees, using FR-HXB2 as root). The numbers near the nodes indicate the percentage of bootstrap replicates (1000). Samples at inclusion: rectal HIV-DNA (open circle), blood HIV-DNA (open triangle), blood HIV-RNA (open square); second set of samples for two patients: rectal HIV-DNA (full circle), blood HIV-DNA (full triangle), blood HIV-RNA (full square). A, B, C, D, E, F and G illustrate viral clones from patient A, B, C, D, E, F and G, respectively. All the clusters identified with the maximum-likelihood approach were confirmed using a neighbor-joining analysis.

**Greater diversity of HIV DNA variants in the rectum compared to variants in the blood
in patients without HAART**

Véronique Avettand-Fenoel* ^{1,2}, Laurent Hocqueloux ³, Michaela Müller-Trutwin ⁴, Thierry Prazuck ³, Adeline Melard ^{1,2}, Marie-Laure Chaix ^{1,2}, Eric Agoute ³, Christophe Michau ⁵, Christine Rouzioux ^{1,2}

¹ AP-HP, Hôpital Necker-Enfants Malades, Laboratoire de Virologie, Paris, 75015, France

² Université Paris Descartes, EA3620.

³ CHR d'Orléans - La Source, Orléans, France.

⁴ Institut Pasteur, Unité de Régulation des Infections rétrovirales, Paris, France.

⁵ CHR Saint-Nazaire, France.

Running head

Rectal HIV reservoir

***Corresponding author**

Véronique Avettand-Fènoël, Laboratoire de Virologie, Hôpital Necker - Enfants Malades,
149 rue de Sèvres, 75015 Paris. Phone number: 33 1 44 49 49 61,
Fax number: 33 1 44 49 49 60 veronique.avettand@nck.aphp.fr

Abbreviations: HAART: highly active antiretroviral treatment, HIV: human immunodeficiency virus, PBMCs: peripheral blood mononuclear cells

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26 The gut-associated lymphoid tissue represents the largest reservoir of HIV-1. Improving
27 knowledge of this reservoir by studying the diversity of viral population is a key step towards
28 understanding the pathogenesis and dynamics of HIV. Obtaining samples is difficult and
29 little information is available on gut viral quasispecies during the course of infection in
30 humans.

31 The aim of the study was to characterize rectal viral strains and their diversity and to
32 investigate the relationships between the rectal tissue reservoir and viral variants in the blood.
33 Phylogenetic analyses were performed on the *env* sequences for rectal HIV DNA, blood HIV
34 DNA and HIV RNA clones, with maximum likelihood and neighbor-joining methods on
35 seven patients. Genetic diversity was assessed.

36 Higher diversity of HIV DNA clones was noted in the rectum compared to blood in 4 out of 5
37 patients without HAART. Viral diversity was present in the rectum from time of the primary
38 infection. Similar degrees of diversity were observed in the rectum and blood during HAART.
39 Rectal and blood HIV variants were interspersed partially or totally in the seven patients. A
40 certain number of rectal HIV DNA clones were clustered together in six patients.

41 These results suggest that variants in the rectum were more heterogeneous than variants in the
42 blood from patients without HAART, probably because the activated milieu of gut-associated
43 lymphoid tissue may provide an improved environment for viral replication, and indicate
44 exchange of viral populations between blood and rectal tissues, reflecting the dynamics of
45 HIV during course of infection.

46 **Key words:** HIV reservoir, gut-associated lymphoid tissue, rectum, phylogeny, diversity

Introduction

The mucosal-associated lymphoid tissue contains 40-60% of the body's lymphocytes [Cerf-Bensussan and Guy-Grand, 1991; Mowat and Viney, 1997]. The gut-associated lymphoid tissue represents the largest reservoir of human immunodeficiency virus type 1 (HIV-1) [Brenchley et al., 2004; Chase et al., 2006; Clayton et al., 1997; Veazey et al., 1998; Veazey and Lackner, 2005]. Although HIV-1 replication decreases in gut-associated lymphoid tissue with Highly Active Antiretroviral Treatment (HAART), HIV RNA and replication-competent HIV-1 can be detected in gut-associated lymphoid tissue of patients under successful HAART, thus confirming that the intestinal mucosa can act as a residual HIV reservoir despite HAART [Belmonte et al., 2007; Poles et al., 2006].

Improving knowledge of this intestinal reservoir is a key step towards the understanding of the pathogenesis of HIV and better assessment of the efficacy of treatment. Studying the diversity of the viral population within-, and analyzing the potential differences between blood and the intestine may provide an insight into the dynamics of HIV-1.

Previous studies on HIV quasispecies have described HIV compartmentalization in various tissues and fluids, including the brain, lung, semen and cervicovaginal secretions [Chomont et al., 2007; Delwart et al., 1998; Korber et al., 1994; van't Wout et al., 1998; Wong et al., 1997; Zhu et al., 1996]. Due to the difficulty of obtaining samples, little information is available on gut viral quasispecies during the course of infection in humans. Colon-derived HIV-1 variants were studied from autopsied samples from a patient who died of AIDS, but these variants were not compared to circulating blood variants [Wang et al., 2001]. Based on *Nef* analysis, Van Marle et al. [2007] showed compartmentalization of the gut HIV reservoir in patients infected chronically prior to HAART. However, results were less clear with reverse transcriptase sequences. Poles et al. [2001] have observed the presence of some HIV RNA variants resistant to non-nucleosidic reverse transcriptase inhibitor in gut-associated

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3 72 lymphoid tissue and in peripheral blood mononuclear cells (PBMCs) which were different
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5 73 from those observed in the plasma. Evidence of cross-infection between gut-associated
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8 74 lymphoid tissue and PBMCs during HAART has been suggested as a possible mechanism for
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10 75 persistence of HIV [Chun et al., 2008].
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15 77 In this context, the objective of the study was to characterize rectal viral populations, to
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17 78 compare their diversity to the diversity of blood variants, in patients receiving treatment or
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19 79 not, and to investigate the relationship between viral variants in the rectum and those
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21 80 circulating in PBMCs and in the plasma. This study was based on rectal biopsies because they
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23 81 are the easiest gut biopsies to obtain in humans.
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29 83 **Study design**
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31 84 *Patients and samples*
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34 85 Seven patients, enrolled in a study described previously [Avettand-Fenoel et al., 2008], were
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36 86 examined. Two of the patients were at the time of the primary infection within four months of
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38 87 the onset of the acute retroviral illness, four patients were infected chronically and one patient
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40 88 had AIDS (Table 1). Blood samples and four punches of rectum including the lamina propria
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42 89 were collected on the same day. In two patients, a second set of samples was taken several
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44 90 months later (B, C) (Table 1). In total, six sets of samples were collected in patients without
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46 91 HAART (A, B first set of samples, C first set of samples, C second set of samples, D, G) and
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48 92 three during HAART (B second set of samples, E, F) (Table 1). Patients A, B (1st sample), C
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50 93 were naïve to HAART; Patients D and G had previously received HAART but were not
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52 94 receiving treatment at the time of sample collection (Table 1). Rectal endoscopic biopsies
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54 95 were undertaken at the same site to avoid potential variations among patients. Rectal tissue
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was also collected from the same site for histopathological assessment. The local ethics committee approved the study and patients gave their written informed consent.

Nucleic acids extraction

RNA was extracted from plasma using the QIAamp RNA Mini Kit (Qiagen, Courtaboeuf, France) and used to synthesize cDNA with the Reverse transcriptase core kit (Eurogentec, Angers, France). Total DNA was extracted from PBMCs using the QIAamp DNA mini kit and from rectal tissues using the QIAamp tissue kit (Qiagen). The extracts of the four rectal biopsies collected on the same day from each patient were pooled.

Cloning and sequencing

The *env* regions encoding C2-V5 were amplified using a previously described nested PCR [Delwart et al., 1993]. Viral sequences were amplified using an Expand High Fidelity^{PLUS} PCR system (Roche Diagnostics, Meylan, France). To avoid a PCR-based bias, at least two PCRs from the pooled extracts were performed on each sample. Rectal HIV DNA, blood HIV DNA and blood HIV RNA from each patient, as well as the sequential samples, were amplified on different days to avoid contamination. To prevent any inter-sample contamination, water and one sample from each patient were amplified on one day. Moreover, the different amplifications of the samples from each patient were separated by at least two weeks.

Phylogenetic analyses

A direct cloning of PCR products was performed as described previously [Briat et al., 2005]. Clones were sequenced separately from each PCR amplification. Sequences were analyzed with Sequence Navigator software and aligned with ClustalX software [Thompson et al., 1997]. Gap-stripped sequence alignments were performed for each patient. Distances between pairs of sequences were estimated based on Kimura's two-parametric model of nucleotide substitution (DNAdist module - PHYLIP package, v3.67) [Felsenstein, 2001]. The means of

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3 121 all comparisons of pairwise genetic distances from each clone derived from rectal HIV DNA,
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5 122 from PBMC HIV DNA or from plasma HIV RNA to every other clone from the same
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8 123 tissue/fluid were calculated for each patient. Maximum-likelihood trees were constructed
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10 124 using the HKY model (PHYML v2.4.4) [Guindon and Gascuel, 2003]. The reliability of the
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13 125 branching orders was confirmed by performing 1,000 bootstrap resamplings. In order to
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15 126 confirm the topology of the resulting trees, the distance matrix was employed for
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17 127 phylogenetic analysis with the neighbor-joining method. Bootstrap values >70% were
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20 128 considered significant.

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22 129 *Statistical considerations*

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24 130 The differences detected between pairwise distances among variants from the rectum, variants
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27 131 from PBMCs and variants from plasma were assessed using Mann-Whitney test.

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31 133 **Results**

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33 134 Seven patients were included: patients A and B were at the time of the primary infection;
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35 135 patients C, D, E, and F at chronic infection, and patient G with AIDS (Table 1). No
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38 136 confounding condition such as inflammation was observed during histopathological analyses
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41 137 of rectal tissue.

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43 138 Overall, phylogenetic analyses were conducted on 522 sequences from clones derived from
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45 139 nine rectal samples, four plasma samples and nine PBMC samples (six to 30 clones per
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48 140 sample, median: 25) (GenBank accession numbers: GQ859192-GQ859245; GQ904239-
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50 141 GQ904706).

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52 142 The phylogenetic analysis showed that all sequences of rectal clones and of blood clones from
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54 143 each patient clustered together, with no contamination between the samples from different
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57 144 patients (bootstrap=100%) (data not shown).

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Pairwise distances within HIV DNA quasiespecies in the rectum, within HIV DNA variants in PBMCs and within HIV RNA variants in the plasma were calculated. These seven patients with diverse history of infection had very different levels of genetic diversity, with medians of pairwise distances within HIV DNA quasiespecies in the rectum ranging from 0.1 to 2.2% depending on the patient taken into account (Fig.1a), from 0.02 to 1.8% within HIV DNA quasiespecies in the PBMCs (Fig.1b) and from 0.04 to 1.95% within HIV RNA quasiespecies in the plasma (data not shown).

The genetic diversity within HIV variants in the rectum was then compared to the diversity within HIV variants in the blood for each patient. There was a correlation between the diversity of HIV DNA variants in the gut and the diversity of HIV DNA variants in PBMCs among the 9 pairs of samples ($R=0.66$, $p=0.05$). Data were presented i) for patients without HAART, ii) for patients with HAART. i) In four out of five patients without HAART, the diversity among HIV DNA variants in the rectum was significantly greater than among HIV DNA variants in PBMCs ($p<0.0001$). Differences between the median distance within variants in the rectum and the median distance within variants in PBMCs are presented in Figure 1 (patients A, B first set of samples, D, G). Surprisingly, this was observed from the primary infection stage (A, B first set of samples). The diversity among HIV DNA variants in the rectum was also significantly greater than among HIV RNA variants in the plasma ($p<0.0001$). Differences between the medians distances within variants in the rectum and within variants in the plasma were +0.07%, +0.46%, +0.31% for the first set of samples of patient B, patient D and patient G respectively.

ii) During HAART, the genetic diversity within HIV DNA variants in the rectum and within HIV DNA variants in PBMCs for each patient was not statistically different ($p=0.14$; $p=0.35$ and $p=0.56$ for patients B (second set of samples), E and F, respectively) (Fig. 1). It was also

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noticeable for patient B that the genetic diversity within HIV DNA quasispecies in the rectum was significantly higher before treatment (median 0.25%) than during HAART (median 0.12%) ($p=0.001$).

Another objective was to check whether the HIV variants in the rectum and in the blood were interspersed or separated. Phylogenetic analyses showed that variants in the rectum and in the blood were interspersed partially or totally in all the seven patients (Fig. 2). In patients with sequential samples, most of the variants from the first set and variants in the blood from the second set were interspersed (B, C), showing the persistence of some archived variants in the body over time (Fig. 2). Nevertheless, clusters grouping at least some variants were observed in the rectum in patients A, B, C, E, F, G (bootstrap values $>70\%$) (Fig. 2). It was also noticeable that all the clusters identified with the maximum likelihood analysis were confirmed using a neighbor-joining approach.

Discussion

Studies carried out on the viral quasispecies in the different tissues could provide useful information enabling understanding of virus trafficking. Very little is known about the gut-associated lymphoid tissue in patients infected with HIV, due to the small size of samples and the difficulty in obtaining such samples. For the first time, the genetic diversity of HIV DNA in the rectum was compared to the genetic diversity of variants in blood, for well-characterized patients either at the time of the primary infection, or during chronic infection and or with AIDS. The HIV DNA variants include archived viral strains accumulated over time, as well as strains from recent infections; while the HIV RNA variants represent recently

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3 195 produced viral particles. Despite the low number of patients, statistically significant
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8 197 These data suggest that variants in the rectum were more heterogeneous than variants in the
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10 198 blood in patients without HAART. To our knowledge, this is the first report to document that
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12 199 a viral genetic diversity seems to exist in the rectum from the time of the primary infection.
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15 200 For patient B (first set of data), the greater genetic diversity was mainly linked to two
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17 201 sequences of HIV DNA that clustered together. Unfortunately, other rectal HIV DNA
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19 202 sequences could not be recovered in patient B due to the low number of CD4+ T cells in
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21 203 relation to the small size of the rectal biopsies, and further studies will be necessary to
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23 204 confirm that point. One limitation of this study could be the bias introduced by the strategy of
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25 205 PCR cloning. However a recent publication again discussed this point and concluded that this
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27 206 method of cloning is relevant if an adequate number of PCR templates is analysed [Jordan et
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29 207 al., 2010]. In the present study, the number of PCR templates was high (table 1), therefore the
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31 208 strategy of PCR cloning could be considered with a limited bias. Although the existence of
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33 209 undetected minor variants which could influence the viral diversity in PBMCs and in the
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35 210 rectum cannot be excluded, the results may be explained by the activated milieu of the gut-
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37 211 associated lymphoid tissue which may provide an improved environment for the replication of
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39 212 HIV. In a larger group of patients [Avettand-Fenoel et al., 2008], we quantified the
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41 213 proportion of CD4+ rectal cells by immuno-staining in available biopsies for some patients
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43 214 and calculated the HIV DNA load per million of CD4+ rectal cells. It was noted that the HIV
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45 215 DNA load per million of CD4+ rectal cells was higher systematically than the HIV DNA
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47 216 level per million PBMCs, as described by Chun et al. [2008], Chomont et al. [2009] and Yukl
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49 217 et al. [2010]. The microenvironment in the gut-associated lymphoid tissue can be explained
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51 218 by the differences in the local immune selection pressures, the HIV-specific immune
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53 219 response, the constraints on viral entry and the replication rates due to the availability of
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3 220 activated target cells. The increased error prone replication would result in greater viral
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8 222 These data suggest that heterogeneity was not significantly different in the rectum and in the
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10 223 blood during HAART, as described previously [Chun et al., 2008]. This observation suggests
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12 224 that HAART played a role in equalizing the dynamics of HIV in the gut-associated lymphoid
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14 225 tissue and PBMCs, although some clusters persisted.
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19 227 In the cases described, the fact that the HIV variants in the rectum, in PBMCs and in the
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21 228 plasma were interspersed underlines that large exchanges of virus particles and/or infected
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23 229 cells occur between these tissues/fluids, as already shown in patients with HAART [Chun et
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25 230 al., 2008]. This is in line with the positive correlation observed between the HIV DNA levels
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27 231 in the rectum and in PBMCs at different stages [Avettand-Fenoel et al., 2008]. As observed
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29 232 previously with the variants of HIV in faeces [van der Hoek et al., 1998] and in the gut-
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31 233 associated lymphoid tissue [van Marle et al., 2010], complete, independent evolution of HIV-
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33 234 1 was not observed in the intestinal tissue.
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36 235 Nevertheless, in most cases, at least some clones of HIV DNA in the rectum could be
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38 236 differentiated from variants in the blood, as described recently in the esophagus, the stomach,
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40 237 the duodenum, the colon and the blood with phylogenetic and drug mutation analysis [van
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42 238 Marle et al., 2010]. This suggests the existence of local replication foci which could play a
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44 239 major role in the immunopathogenesis of HIV-1 in the altered mucosa.
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52 241 Finally, these results indicate exchange and trafficking of viral populations between blood and
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54 242 lymphoid rectal tissues. The results also suggest that viruses present in the rectum were more
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56 243 heterogeneous than variants in the blood in patients without HAART. Viral diversity is
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58 244 present in the rectum from the time of the primary infection. Studies on the gut-associated
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lymphoid tissue may improve understanding the pathogenesis of HIV, the assessment of treatment efficacy and the development of new therapeutic strategies, particularly those aiming at reducing the reservoir.

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The authors have declared no conflict of interest in this publication.

Ethical approval

The local ethics committee (Tours, France) approved the study and patients gave their written informed consent.

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