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Evolution of HIV-1 tropism at quasispecies level after 5 years of combination antiretroviral therapy in patients always suppressed or experiencing episodes of virological failure

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Objectives: Tropism evolution of HIV-1 quasispecies was analysed by ultra-deep pyrosequencing (UDPS) in patients on first-line combination antiretroviral therapy (cART) always suppressed or experiencing virological failure episodes.

Methods: Among ICONA patients, two groups of 20 patients on cART for ≥5 years, matched for baseline viraemia and therapy duration, were analysed [Group I, patients always suppressed; and Group II, patients experiencing episode(s) of virological failure]. Viral tropism was assessed by V3 UDPS on plasma RNA before therapy (T0) and on peripheral blood mononuclear cell proviral DNA before–after therapy (T0-T1), using geno2pheno false positive rate (FPR) (threshold for X4: 5.75). For each sample, quasispecies tropism was assigned according to X4 variant frequency: R5, <0.3% X4; minority X4, 0.3%–19.9% X4; and X4, ≥20% X4. An R5–X4 switch was defined as a change from R5/minority X4 in plasma/proviral genomes at T0 to X4 in provirus at T1.

Results: At baseline, mean FPR and %X4 of viral RNA were positively correlated with those of proviral DNA. After therapy, proviral DNA load significantly decreased in Group I; mean FPR of proviral quasispecies significantly decreased and %X4 increased in Group II. An R5–X4 switch was observed in five patients (two in Group I and three in Group II), all harbouring minority X4 variants at T0.

Conclusions: UDPS analysis reveals that the tropism switch is not an 'on–off' phenomenon, but may result from a profound re-shaping of viral quasispecies, even under suppressive cART. However, episodes of virological failure seem to prevent reduction of proviral DNA and to accelerate viral evolution, as suggested by decreased FPR and increased %X4 at T1 in Group II patients.

Keywords: cART, HIV quasispecies, co-receptor usage, ultra-deep pyrosequencing, tropism switch

Introduction

In order to initiate infection, HIV-1 requires the primary receptor, CD4, and a secondary receptor (CCR5 or CXCR4). Early in primary infection, HIV-1 strains using the CCR5 co-receptor (called CCR5-using or R5) tend to predominate, suggesting that they are selected during or shortly after transmission. In up to 70% of individuals infected with subtype B HIV-1, either CXCR4-using (X4) or R5X4 (dual/mixed) viruses appear in later stages of the infection,[1–5] and their presence may correlate with subsequent disease progression[6,7] and decrease in CD4, also in the presence of effective therapy.[8] In addition, in antiretroviral-experienced patients X4 variants are more frequent than in chronically infected drug-naive subjects and recent seroconverters.[9–13]

However, in each infected individual, HIV-1 is present as a swarm of highly related variants (referred to as quasispecies), so viral tropism should be better analysed taking into account all the variants present in the quasispecies, each of which has its own

tropism. Ultra-deep pyrosequencing (UDPS) provides a quantitative evaluation of variants in viral quasispecies and permits the relative frequency of even rare variants with co-receptor usage different from that of the predominant virus population to be highlighted and counted.[14–20] Studies performed with UDPS have shown that, in most patients, X4 variants coexist with R5 viruses, even during or shortly after primary infection.[17]

Maraviroc, a licensed CCR5 antagonist, is generally associated with low toxicity. In virologically suppressed patients, this inhibitor may be considered a valid alternative to more toxic antiretrovirals, if viral tropism, assessed on proviral DNA at the time of therapy change or on viral RNA before initial therapy, rules out the presence of X4 variants. The latter option is based on the hypothesis that viral tropism is stable during effective combination antiretroviral therapy (cART). However, while some studies showed that a tropism switch is a relatively rare event under suppressive therapy,[21–25] several lines of evidence have challenged this assumption. In fact other studies reported that a gradual emergence of X4 variants may occur despite effective cART.[22,26–29] These studies have been performed using mainly population sequencing of viral RNA or proviral DNA, providing information only on the average or predominant component(s) of HIV-1 quasispecies.

The aim of the present study was to investigate by UDPS the dynamics of co-receptor usage of HIV-1 quasispecies in patients who had started their first cART and remained under therapy for ≥5 years, either showing always suppressed viraemia or experiencing episode(s) of virological failure. Longitudinal analysis of quasispecies was performed on samples obtained before therapy (T0) and after ≥5 years of therapy (T1).

Methods

Ethics statement

The study was performed entirely on samples collected from patients enrolled in the Italian Cohort of Naive Antiretroviral (ICONA) Foundation Study. A detailed description of the ICONA Foundation Study has been described elsewhere.[30] In brief, it is a prospective study of 9313 HIV-infected patients, naive to antiretrovirals, recruited in 71 Italian clinical sites, 50 of which still provide new enrolments and updated follow-up of the persons enrolled. Data of the patients enrolled (including CD4+ cell counts, viral load, clinical and treatment information) are collected prospectively at clinical sites on at least a 6 monthly basis. All patients signed consent forms to participate in the ICONA Foundation Study, in accordance with the ethical standards of the committee on human experimentation and the Helsinki Declaration (1983 revision). The study was approved by the Ethics Committee of each of the participating institutions, which are listed at the end of the manuscript.

Patients

Samples from patients enrolled in the ICONA Foundation Study were retrospectively selected, in order to fulfil the following conditions: having been on cART (not including CCR5 antagonist) for ≥5 years and showing suppressed HIV-1 viraemia (HIV RNA <50 copies/mL) after this time interval. Among these, two groups of 20 patients, matched for HIV RNA at baseline and for duration of follow-up on cART, were further identified. Group I included patients in whom viral load was always <50 copies/mL during the T0–T1 time lapse (testing interval, ≤6 months). Group II included patients who had experienced at least one virological failure episode during the considered time interval (defined as two consecutive HIV RNA ≥1000 copies/mL) after ≥1 year from first cART start (range of episode failure number, 1–3). For each patient, two timepoints were analysed, the first one prior to initiation of cART (T0) and the second one after ≥5 years (60–113 months) from cART initiation (T1).

Samples

Plasma and peripheral blood mononuclear cell (PBMC) samples, collected from the participating centres and stored in the ICONA Foundation biorepository, were used in the study. Plasma RNA was isolated using the extraction kit included in the Abbott real-time HIV assay (Abbott Molecular Inc., IL, USA). Total DNA was isolated from PBMCs using the 'DNA blood' extraction kit (Qiagen, Hilden, Germany). Total proviral HIV-1 DNA (here referred to as proviral DNA) was evaluated using a quantitative real-time PCR targeting the LTR region, as described previously.[31]

Sequencing

Conventional sequencing was performed with the ABI Prism 310 instrument, using the BigDye Terminator cycle sequencing kit (Applied Biosystems). UDPS was performed with the 454 Life Sciences platform (GS-FLX, Roche Applied Science, Monza, Italy), using Titanium chemistry.[15,17] V3 amplification was performed by nested PCR. Briefly, two rounds of 30 cycles (94°C for 2 min, 94°C for 30 s, annealing at 60°C for 30 s, extension at 68°C for 30 s and final elongation at 68°C for 5 min) were carried out using a proof-reading DNA polymerase (Platinum® Taq DNA Polymerase High Fidelity, Invitrogen, by Life Technologies, Monza, Italy). First- and second-round primers were described previously.[15] For plasma samples, the first round of V3 included a one-step RT–PCR, using a Platinum quality proof-reading reverse transcriptase (Invitrogen). Unique in-house-designed stretches of eight nucleotides (multiplex identifiers) were used to tag each sample. To maximize the genetic heterogeneity of viral genomes to be amplified and sequenced, and to overcome problems of template resampling, for each sample the amplicons from at least four replicate PCRs were pooled. The total number of templates undergoing UDPS, resulting from the pooling of four replicate reactions, represented the content of 1 mL of plasma for viral RNA, and the content of $2–6\times10^6$ PBMCs for proviral DNA, with a minimum of 3200 genome templates undergoing UDPS. For three patients from Group II, a smaller number of HIV DNA templates (438, 258 and 1086 copies) at T1 could undergo UDPS, due to a low content of proviral DNA. The correction pipeline and the evaluation of experimental error necessary to establish the sensitivity threshold have already been described.[15] Since no correlation between input copy number and X4 frequency at both RNA and DNA levels was observed (i.e. $\rho=0.084$ and -0.067, $P=0.642$ and 0.700 for RNA and DNA at T0, respectively), input copy number was not considered in the correction pipeline. Considering the number of viral templates actually undergoing UDPS and the corrected error rate, the threshold of sensitivity for V3 was set at 0.3%;[16,17] this threshold was used for the assignment of quasispecies tropism (see below).

The nucleotide sequences resulting from the correction pipeline were also analysed to establish genetic heterogeneity of the viral quasispecies (diversity). For each sample, mean substitutions/site were calculated using DNADIST (F84 algorithm, Phylip package). All of the V3 sequences generated for this study through conventional sequencing or UDPS are available on request.

Tropism assessment

HIV-1 genotypic tropism assessment was based on V3 sequencing. In more detail, the genotypic predictor algorithm geno2pheno was applied to the V3 nucleotide sequences obtained by conventional sequencing and by UDPS. Differently from the MOTIVATE studies, where different false positive rate (FPR) cut-offs were used for tropism assignment in conventional and UDPS analysis,[32] we decided to adopt a unique cut-off for

conventional and ultra-deep analysis (5.75%), applied to both proviral DNA and viral RNA genomes. For UDPS analysis, FPR was established for each variant, then the average FPR and %X4 were calculated for each individual sample. Quasispecies tropism was assigned to individual samples according to the frequency of X4 variants: R5, <0.3% X4; minority X4, 0.3%–19.9% X4; and X4, ≥20% X4. An R5–X4 tropism switch was defined as change of classification from R5/minority X4 in both plasma and proviral genomes at T0 to X4 in provirus at T1. About 20% of samples were omitted from the analysis due to failure to amplify the V3 region, bad quality sequencing results or insufficient material for performing both conventional sequencing and UDPS.

Statistical analysis

The viro-immunological parameters were compared using a non-parametric Mann–Whitney test or by a paired or unpaired Student's *t*-test, as appropriate. For calculations, the lowest detectable values were assigned to the samples with values below the detection threshold. For correlation analysis, the Spearman rank correlation coefficient (ρ) was calculated. *P* values <0.05 were considered statistically significant.

Results

Baseline patient characteristics and tropism changes assessed by conventional sequencing and by UDPS after ≥5 years of cART

At baseline, the two groups of patients did not significantly differ for proviral load as well as for current and nadir CD4 T cell count (Table 1). In Group II, the time from HIV diagnosis was higher, although not significantly, than in Group I; median duration of

Table 1. Clinical and virological characteristics of study patients

	Group I	Group II	P^a
Patients, *n*	20	20	
Age (years), median (range)	38 (24–46)	40 (32–52)	0.3102
Male, %	70	70	NA
Time from HIV diagnosis (weeks), median (range)	47 (2–871)	137 (2–872)	0.3720
Baseline CD4 (cells/mm^3), median (range)	420 (6–1256)	403 (14–1145)	0.8610
Nadir CD4 (cells/mm^3), median (range)	352 (6–1081)	398 (14–750)	0.6197
Baseline viral load ($\log_{10}$ HIV RNA copies/mL), median (range)	4.9 (4.0–6.2)	4.8 (4.1–6.1)	0.6947
Baseline proviral load ($\log_{10}$ HIV-1 DNA copies/10^6 cells), median (range)	4.1 (3.2–4.8)	4.2 (3.3–5.4)	0.7895
Duration of virological failure (months), median (range)	NA	16 (1–77)	NA
Peak viral load during failure ($\log_{10}$ HIV RNA copies/mL), median (range)	NA	5.3 (4.1–6.0)	NA

NA, not applicable.
Group I, patients who received cART for ≥5 years, in whom viral load was always <50 copies/mL.
Group II, patients who received cART for ≥5 years and experienced at least one episode of virological failure while on cART during the observation period.
[a]Mann–Whitney test

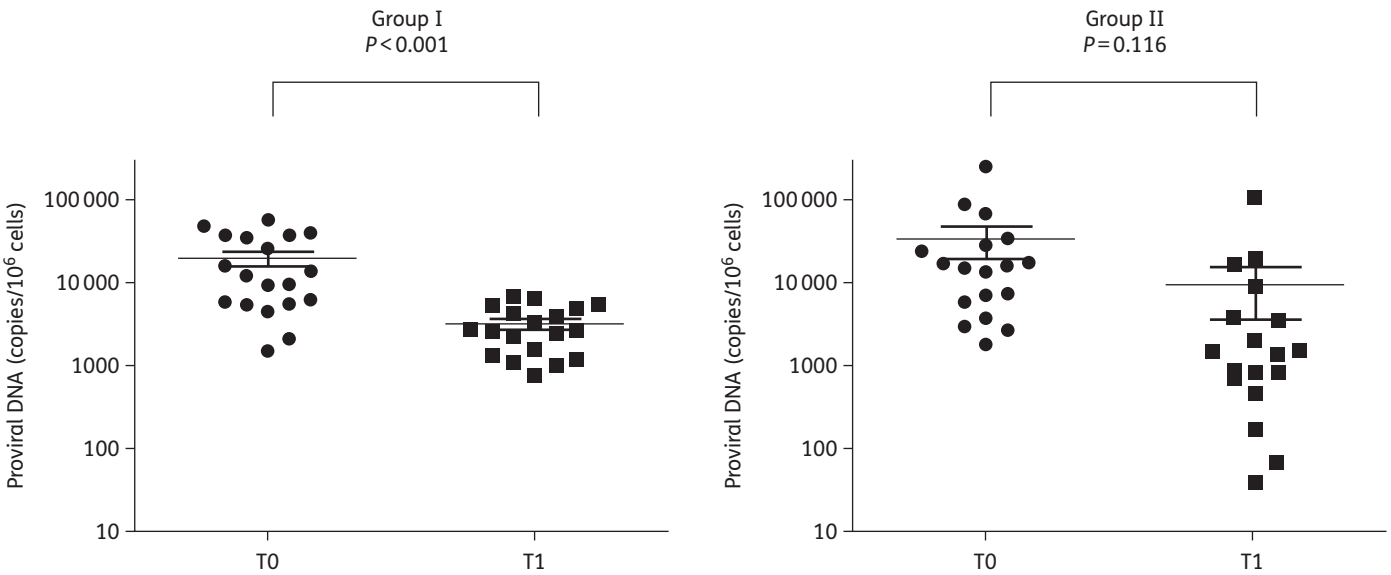

Figure 1. HIV-1 proviral DNA load in PBMCs from Group I and Group II patients at T0 and T1. Results are expressed as HIV-1 DNA copies/10^6 cells. Means and standard deviations are shown, and comparison was performed using a paired Student's *t*-test.

Table 2. FPR and tropism classification of circulating plasma HIV RNA and PBMC-associated proviral DNA at baseline (T0) and after ≥5 years of cART (T1) in the two groups of patients, by conventional population sequencing

	Plasma virus T0		Proviral DNA T0		Proviral DNA T1	
Patient ID	FPR	tropism classification (X4/R5)	FPR	tropism classification (X4/R5)	FPR	tropism classification (X4/R5)
Group I						
I-1	56.7	R5	44.2	R5	NA	NA
I-2	3.7	X4	19.1	R5	3.7	X4
I-3	11.4	R5	70.7	R5	NA	NA
I-4	66.0	R5	71.4	R5	NA	NA
I-5	NA	NA	91.0	R5	24.0	R5
I-6	4.0	X4	0.7	X4	NA	NA
I-7	4.1	X4	NA	NA	NA	NA
I-8	34.6	R5	50.2	R5	NA	NA
I-9	31.7	R5	42.6	R5	20.7	R5
I-10	62.4	R5	62.4	R5	NA	NA
I-11	NA	NA	48.4	R5	NA	NA
I-12	35.3	R5	35.3	R5	31.4	R5
I-13	83.0	R5	42.6	R5	17.0	R5
I-14	7.1	R5	7.1	R5	7.1	R5
I-15	**12.0**	**R5**	**8.5**	**R5**	**3.2**	**X4**
I-16	55.5	R5	NA	NA	57.0	R5
I-17	11.5	R5	44.2	R5	NA	NA
I-18	31.4	R5	31.4	R5	NA	NA
Median	31.5		43.4		18.9	
Range	3.7–83.0		0.7–91.0		3.2–57.0	
IQR	48.2		37.2		25.0	
Group II						
II-1	NA	NA	6.8	R5	NA	NA
II-2	84.9	R5	84.9	R5	68.6	R5
II-3	70.7	R5	70.7	R5	70.7	R5
II-4	34.9	R5	27.3	R5	24.7	R5
II-5	**68.3**	**R5**	**63.1**	**R5**	**1.1**	**X4**
II-6	62.4	R5	62.4	R5	NA	NA
II-7	0.5	X4	14.8	R5	NA	NA
II-8	26.9	R5	42.3	R5	18.3	R5
II-9	10.1	R5	20.5	R5	8.5	R5
II-10	47.8	R5	47.8	R5	47.8	R5
II-11	**16.2**	**R5**	**16.2**	**R5**	**1.3**	**X4**
II-12	39.4	R5	39.4	R5	39.4	R5
II-13	**29.8**	**R5**	**37.8**	**R5**	**0.0**	**X4**
II-14	36.2	R5	31.7	R5	60.5	R5
II-15	44.2	R5	44.2	R5	44.2	R5
II-16	NA	NA	NA	NA	33.9	R5
II-17	1.1	X4	1.1	X4	1.1	X4
II-18	1.8	X4	0.5	X4	0.2	X4
II-19	NA	NA	92.0	R5	67.3	R5
Median	35.5		38.6		29.3	
Range	0.5–84.9		0.5–92.0		0.0–70.7	
IQR	47.1		46.7		56.1	
P^a	0.706		0.593		0.603	

NA, not available.
The R5–X4 switches are shown in bold.
[a]Statistical significance between Group I and Group II, Mann–Whitney test.

Table 3. Genetic heterogeneity and quasipecies tropism (%X4 variants, mean FPR and tropism classification) of circulating plasma HIV RNA and PBMC-associated proviral DNA at baseline (T0) and ≥5 years of cART (T1), by UDPS

	Plasma virus T0				Proviral DNA T0				Proviral DNA T1			
Patient ID	%X4	mean FPR	tropism classification X4/R5	diversity	%X4	mean FPR	tropism classification X4/R5	diversity	%X4	mean FPR	tropism classification X4/R5	diversity
Group I												
I-1	<0.3	56.3	R5	0.0130	7.5	36.7	minority X4	0.0911	NA	NA	NA	NA
I-2	71.9	13.9	X4	0.0438	20.1	24.6	X4	0.1539	93.5	6.7	X4	0.0156
I-3	1.8	10.6	minority X4	0.0116	3.8	52.2	minority X4	0.1064	NA	NA	NA	NA
I-4	<0.3	65.0	R5	0.0225	<0.3	64.5	R5	0.0202	NA	NA	NA	NA
I-5	<0.3	43.1	R5	0.0673	<0.3	42.1	R5	0.0771	11.7	47.7	minority X4	0.1184
I-6	86.3	6.5	X4	0.0289	97.0	1.0	X4	0.0336	NA	NA	NA	NA
I-7	95.1	5.3	X4	0.0292	42.1	33.7	X4	0.1305	NA	NA	NA	NA
I-8	0.3	44.6	minority X4	0.0378	10.9	36.4	minority X4	0.1036	NA	NA	NA	NA
I-9	0.5	30.7	minority X4	0.0132	2.6	54.1	minority X4	0.0692	<0.3	20.6	R5	0.0024
I-10	<0.3	63.2	R5	0.0056	<0.3	63.0	R5	0.0131	NA	NA	NA	NA
I-11	NA	NA	NA	NA	0.7	48.0	minority X4	0.0148	NA	NA	NA	NA
I-12	2.1	33.2	minority X4	0.0241	4.1	32.4	minority X4	0.0283	0.6	24.8	minority X4	0.0339
I-13	<0.3	66.8	R5	0.0293	12.2	46.2	minority X4	0.0973	<0.3	23.0	R5	0.0128
I-14	**2.2**	**7.2**	**minority X4**	**0.0045**	**1.5**	**7.0**	**minority X4**	**0.0025**	**25.1**	**5.4**	**X4**	**0.0140**
I-15	**11.4**	**12.2**	**minority X4**	**0.0383**	**7.25**	**14.2**	**minority X4**	**0.0343**	**52.0**	**12.3**	**X4**	**0.0365**
I-16	<0.3	54.7	R5	0.0151	13.0	35.2	minority X4	0.1477	6.6	49.8	minority X4	0.0363
I-17	2.1	11.8	minority X4	0.0040	7.4	46.4	minority X4	0.0859	0.3	19.1	minority X4	0.0105
I-18	5.9	30.4	minority X4	0.0257	17.0	26.5	minority X4	0.0664	NA	NA	NA	NA
Median	1.8	30.7		0.0241	7.3	36.6		0.0732	6.7	20.6		0.0156
Range	<0.3–95.1	5.3–66.8		0.0040–0.0673	<0.3–96.1	1.0–64.5		0.0025–0.1539	<0.3–93.5	5.4–49.8		0.0024–0.1184
IQR	8.4	44.3		0.0213	12.7	23.0		0.0780	38.2	26.8		0.0248
Group II												
II-1	NA	NA	NA	NA	0.6	7.5	minority X4	0.0156	NA	NA	NA	NA
II-2	<0.3	83.9	R5	0.0120	<0.3	84.3	R5	0.0060	<0.3	67.5	R5	0.0183
II-3	0.7	71.1	minority X4	0.0095	<0.3	72.1	R5	0.0027	<0.3	71.8	R5	0.0019
II-4	<0.3	34.4	R5	0.0121	<0.3	36.4	R5	0.0712	<0.3	24.8	R5	0.0025
II-5	**7.8**	**59.3**	**minority X4**	**0.0533**	**3.3**	**62.9**	**minority X4**	**0.0511**	**53.1**	**28.2**	**X4**	**0.0875**
II-6	<0.3	61.5	R5	0.0110	<0.3	58.6	R5	0.0143	NA	NA	NA	NA
II-7	62.2	8.5	X4	0.0487	0.9	24.5	minority X4	0.0456	99.5	0.4	X4	0.0468
II-8	<0.3	36.7	R5	0.0374	<0.3	45.6	R5	0.0394	<0.3	27.3	R5	0.0285
II-9	1.4	9.7	minority X4	0.0141	0.4	21.3	minority X4	0.0167	1.7	8.0	minority X4	0.0226
II-10	<0.3	46.2	R5	0.0103	<0.3	46.1	R5	0.0090	<0.3	45.9	R5	0.0289
II-11	**1.0**	**14.6**	**minority X4**	**0.0196**	**0.4**	**16.8**	**minority X4**	**0.0226**	**84.6**	**4.8**	**X4**	**0.0334**
II-12	6.5	34.9	minority X4	0.0307	11.5	34.2	minority X4	0.0384	<0.3	32.6	R5	0.0277
II-13	**1.0**	**23.6**	**minority X4**	**0.0951**	**<0.3**	**45.5**	**R5**	**0.0233**	**97.4**	**1.3**	**X4**	**0.0165**
II-14	<0.3	39.4	R5	0.0181	<0.3	40.4	R5	0.0184	1.1	50.9	minority X4	0.0215

Continued

Table 3. *Continued*

Patient ID	Plasma virus T0				Proviral DNA T0				Proviral DNA T1			
	%X4	mean FPR	tropism classification X4/R5	diversity	%X4	mean FPR	tropism classification X4/R5	diversity	%X4	mean FPR	tropism classification X4/R5	diversity
II-15	0.5	43.1	minority X4	0.0041	0.5	42.9	minority X4	0.0078	<0.3	48.8	R5	0.0056
II-16	NA	NA	NA	NA	NA	NA	NA	NA	<0.3	31.2	R5	0.0114
II-17	53.1	11.7	X4	0.0573	68.3	8.3	X4	0.0486	65.0	11.4	X4	0.0778
II-18	49.9	20.7	X4	0.0832	79.9	9.6	X4	0.0419	96.8	1.3	X4	0.0275
II-19	NA	NA	NA	NA	0.5	92.2	minority X4	0.0229	<0.3	67.1	R5	0.0166
Median	0.8	35.8		0.0189	0.4	41.6		0.0228	<0.3	28.2		0.0226
Range	<0.3–62.2	8.5–83.9		0.0041–0.0951	<0.3–79.9	7.5–92.2		0.0027–0.0712	<0.3–99.5	0.4–71.8		0.0019–0.0875
IQR	7.2	39.9		0.0409	1.2	39.6		0.0299	75.5	43.5		0.0172
P^a	0.726	0.482		0.732	0.012	0.788		0.008	0.654	0.484		0.957

NA, not available.
The R5–X4 switches are shown in bold.
[a]Statistical significance between Group I and Group II, Mann–Whitney test.

virological failure episodes was 16 (range, 1–77) months; median value of HIV RNA peak reached in failure episode(s) was 5.3 (range, 4.1–6.0) $\log_{10}$ HIV RNA copies/mL (Table 1).

Considering all patients, plasma viraemia (HIV-1 RNA) and PBMC-associated proviral DNA load at baseline were positively correlated ($\rho=0.49$, $P=0.002$); nadir CD4 was inversely correlated with T0 proviral load ($\rho=-0.34$, $P=0.041$) and with %X4 in T0 DNA ($\rho=-0.36$, $P=0.038$). Mean proviral load tended to decrease between T0 and T1, reaching statistical significance in Group I (from 19732 to 3186 copies/10^6 cells, $P<0.001$, paired *t*-test) but not in Group II (from 33885 to 9715 copies/10^6 cells, $P=0.116$) (Figure 1).

Co-receptor usage of baseline plasma HIV (T0) and PBMC provirus (T0 and T1), assessed by conventional sequencing, is shown in Table 2. Pairwise comparison between T0 and T1 was obtained for 22 patients. In the considered time interval, a tropism switch (from R5 to X4) occurred in four patients, including one patient from Group I (I-15) and three patients from Group II (II-5, II-11 and II-13).

A detailed analysis of viral tropism at quasispecies level was obtained with UDPS. A total of 225602 (median, 6547; range, 2210–14187 sequences/patient) and 348011 (median, 9016; range, 1673–20630 sequences/patient) V3 sequences from plasma HIV-1 RNA and proviral DNA were obtained from T0 samples, respectively, and 264505 (median, 8781; range, 1744–19298 sequences/patient) proviral sequences were obtained from T1 samples.

Considering all samples, quasispecies diversity was positively correlated with %X4 ($\rho=0.53$, $P<0.0001$). Mean FPR and %X4 of T0-RNA were positively correlated with those of T0-DNA ($\rho=0.74$, $P<0.0001$ for mean FPR; $\rho=0.66$, $P<0.0001$ for %X4).

Intra-patient X4 frequencies in plasma viral RNA (T0) and proviral DNA (T0 and T1), together with the mean FPR, tropism classification and genetic diversity of the V3 quasispecies, are reported in Table 3. Pairwise comparison between T0 and T1 was obtained for 25 patients. According to the tropism classification at quasispecies level (see the Methods section), the proportion of patients infected with R5, minority X4 or X4 viruses was 36%, 45% and 18% in T0-RNA, 31%, 56% and 14% in T0-DNA and 44%, 24% and 36% in T1-DNA, respectively. Among the 25 patients with pairwise UDPS comparison, 9 showed a decrease, 5 showed stable values and 11 showed an increase in X4 frequency in proviral genomes. The decrease in proviral DNA load in patients who showed an increase in %X4 was similar to that observed in the patients with stable or decreasing %X4 (median change, −9080; range, −34113–81337 copies/10^6 cells versus median change, −13479; range, −232801–1247 copies/10^6 cells, $P=0.427$).

A tropism switch based on UDPS results was observed in five patients, including all those identified by conventional sequencing, plus one additional patient from Group I (I-14), who at T1 was classified as R5 by population sequencing and X4 by UDPS (X4 frequency, 25.1%).

Changes in proviral quasispecies parameters

Changes in the viral diversity of proviral quasispecies after ≥5 years of cART for Group I and Group II patients are shown in Figure 2. The results indicate a tendency towards a reduction in viral diversity only in Group I, although without reaching statistical

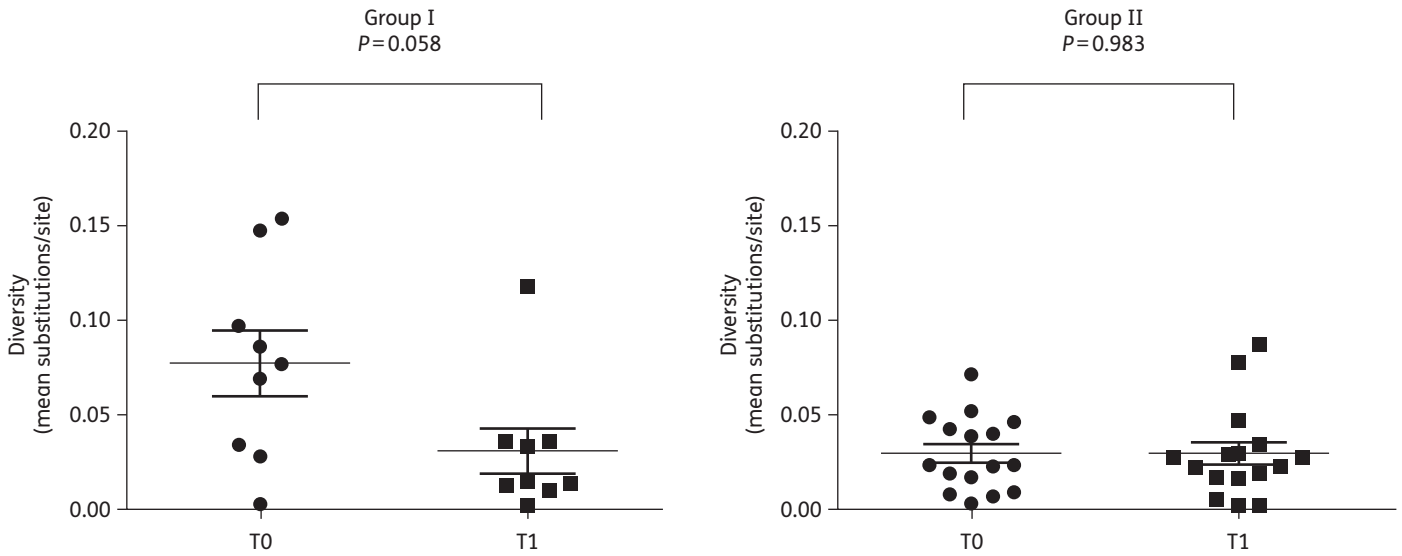

Figure 2. V3 diversity in proviral DNA in patients from Group I and Group II at T0 and T1. Results are expressed as mean substitutions/site. Means and standard deviations are shown, and T0 and T1 comparison was performed using a paired Student's *t*-test. Mean values of diversity were significantly higher in Group I patients (0.0774 versus 0.0291, $P=0.0026$ in unpaired *t*-test) at T0, while there was no difference at T1 (0.0312 versus 0.0290, $P=0.8550$ in unpaired *t*-test).

significance ($P=0.058$), and possibly resulting from higher diversity levels at T0.

The distribution at T0 and T1 of mean FPR (a and b) and X4 frequency (c and d) for Group I and Group II is shown in Figure 3. A decrease in mean FPR after ≥5 years of cART was observed in both groups, but statistical significance was reached in Group II (42.7 versus 30.8, $P=0.006$), while only a trend was observed in Group I (33.6 versus 23.3, $P=0.092$). In Group II, the extent of FPR changes was highly correlated with the highest HIV RNA levels reached during virological failure episode(s) ($\rho=0.67$, $P=0.004$). In agreement with FPR data, a significant increase in %X4 from T0 to T1 was observed in Group II (10.5 versus 31.4, $P=0.046$), while only a trend was observed in Group I (7.6 versus 21.2, $P=0.196$). Neither T0 nor T1 proviral load was significantly different in the 11 patients who showed an increase in %X4 as compared with the 14 patients with stable or decreasing %X4 (not shown), but FPR in T0 proviral genomes was significantly lower (median, 24.5; range, 7.0–62.9 versus median, 45.9; range, 8.3–92.2, $P=0.017$); in the five patients showing a R5–X4 switch, the FPR of T0 proviral DNA was even lower (median 14.6; range, 7.2–59.3). In all of these five patients, minority X4 variants were shown at T0 in both HIV DNA and RNA ($n=4$) or only in RNA ($n=1$). However, nine patients harbouring minority X4 at T0 did not show a tropism switch at T1.

Discussion

The main purpose of the study was to evaluate HIV-1 tropism dynamics at a quasispecies level in a cohort of patients enrolled as naive, with suppressed viraemia after ≥5 years of cART. These patients belonged to two groups: Group I with viral load always suppressed; and Group II having experienced at least one episode of virological failure during the considered time interval. The results indicated that 45%, 56% and 24% of patients harboured minority X4 variants (with a frequency between 0.3% and 19.9%) in T0-RNA, T0-DNA and T1-DNA, respectively. All of the patients with minority X4 were classified as R5 by conventional sequencing (Table 2). A general trend towards an increase in X4 frequency and a decrease in mean FPR in proviral quasispecies after ≥5 years of treatment was observed, but the change reached statistical significance only in the group of patients who experienced episode(s) of virological failure during the considered time interval. On the other hand, a general trend towards decreased proviral load was observed, but the change was statistically significant only in patients who showed constant virological suppression. It is possible that the lack of reduction in Group II patients was due to the longer time from HIV diagnosis (Table 1). Among the patients for whom pairwise comparison was possible, 5 (2 from Group I and 3 from Group II) out of 25 displayed an R5–X4 tropism switch during the observation time, determined by UDPS; in fact, a switch in 1 patient (who reached 25.1% X4 at T1) was not recognized by population sequencing, owing to lower sensitivity in detecting minority variants.

Direct viral population sequencing on plasma and PBMC samples is currently accepted as an appropriate method for predicting co-receptor usage, due to its simplicity and acceptable correlation with clonal analysis.[33] Several studies based on direct sequencing have indicated that, during stable viral suppression, a tropism switch is rare and without preferential direction. In fact, an average 10% expected rate of genotypic tropism switch from the last measurement in plasma RNA before treatment and the follow-up test on PBMC DNA after at least 1 year of successful treatment has been reported.[21,23,25] The rate of switching was lower (about 6%) when comparing tropism assessed on proviral DNA collected longitudinally.[25] Importantly, none of these studies documented any preferential direction of tropism switch, resulting in around 5% of R5 to X4/DM changes. In a few patients with virological failure, alternative switches (R5–X4–R5 or X4–R5–X4) were also reported.[34] Substantially similar results were obtained in a recent

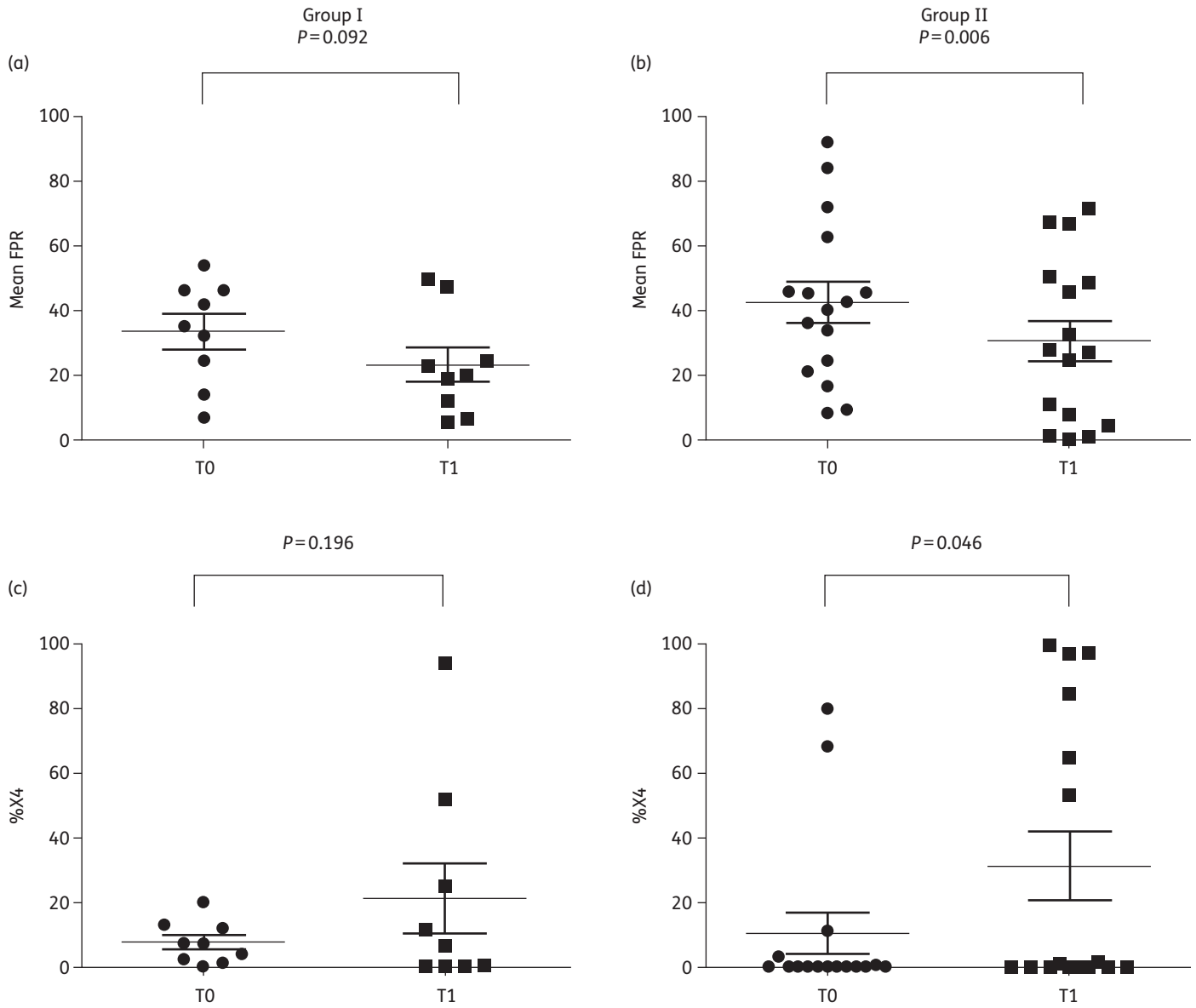

Figure 3. Distribution of FPR (a and b) and X4 frequencies (c and d) in proviral DNA in patients from Group I and Group II at T0 and T1. Results are expressed as mean FPR and %X4. Means and standard deviations are shown, and comparison was performed using a paired Student's *t*-test.

study performed on ICONA patients, with bidirectional viral tropism switch observed in about 18% (R5–X4 switch observed in 10%) of patients sampled longitudinally in a time interval of about 18 months under cART.[27] On the whole, these studies were performed using population sequencing, which is unable to recognize variants with a frequency below 10%–20%, and is therefore prone to underestimate co-existing R5 and X4 variants for mere technical reasons.

In our study, while several patients with minority X4 variants at baseline did not switch during follow-up, all switching patients harboured minority X4 variants (in plasma or PBMCs) at baseline, suggesting that these variants may represent the seeding viral subpopulations, which are subsequently enriched, leading to a switch.

One limitation of the present study is the small sample size. In fact, the long observation time (60–113 months) may be too wide for such a small number of patients. However, despite this limitation, the findings reported here are in substantial agreement with a recent study performed with UDPS in untreated patients, where a 7.6% tropism switch rate was reported over about 30 months. In fact, in this study, the outgrowth of a minority X4 strain was observed over time in about 20% of patients who had experienced an R5–X4 switch. In addition, the low FPR observed in our study at T0 in patients who switched is in line with the results from this study, where FPR <50 at diagnosis was a strong predictor of an R5–X4 tropism switch.[35]

In conclusion, it appears that the HIV-1 tropism switch is not an 'on–off' phenomenon, but rather results from a profound

re-shaping of viral quasispecies, which may occur even in patients with constantly suppressed viraemia. In addition, in the patients described, those who experienced some episodes of virological failure seemed to display an accelerated evolution of viral quasispecies, as proved by a decrease in FPR and an increase in %X4 during the observation period. The positive correlation between the highest levels of HIV RNA reached during virological failure episode(s) and the extent of FPR decrease at T1 in Group II patients strongly supports this point.

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Transparency declarations

None to declare.

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