Co-receptor switch during HAART is independent of virological success

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Co-receptor switch during HAART is independent of virological success

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Table I. Shift of V3 genotype and MT-2 phenotype according to viro-immunological response to HAART.

Baseline patient characteristics							Virological and immunological outcome			Co-receptor Shift		
Pt	MT-2 isolate	RNA V3	DNA V3	Baseline CD4	Baseline pVL	Antiretroviral Therapy	New HAART regimen	F-up CD4	F_up pVL	MT-2 isolate	RNA V3	DNA V3
Naive patients												
1	SI	R5	X4	48(6%)	5,56	none	EFV +AZT+3TC	169 (19%)	1,7	—	—	X4→R5
2	SI	R5	X4	9 (1%)	5,07	none	LPV/r + TDF+3TC	198 (13%)	1,7	—	—	X4→R5
3	NSI	X4/R5	X4	94 (4%)	2,66	none	LPV/r + TDF+3TC	104 (6%)	1,7	—	—	X4→R5
4	NSI	R5	X4/R5	590 (24%)	5,14	none	LPV/r + TDF/FTC+T-20	633 (24%)	1,7	—	—	X4/R5→R5
5	NSI	R5	R5	3 (1%)	5,00	none	LPV/r + TDF/FTC	170 (10%)	1,7	—	—	R5→X4
6	NSI	R5	R5	15 (3%)	5,32	none	EFV +ABC/3TC	112 (8%)	1,7	—	—	R5→X4
7	NSI	X4	R5	22 (4%)	4,60	none	LPV/r + ABC/3TC	369 (16%)	1,7	—	—	no
8	NSI	R5	R5	72 (12%)	4,54	none	LPV/r + ABC/3TC	395 (26%)	1,7	—	—	no
9	NSI	R5	R5	73 (6%)	4,68	none	LPV/r + TDF/FTC	341 (11%)	1,7	—	—	no
10	NSI	R5	X4/R5	13 (3%)	5,25	none	LPV/r + ABC/3TC	364 (12%)	1,7	—	—	no
11	NSI	R5	R5	392 (24%)	4,90	none	LPV/r + ABC/3TC	484 (29%)	1,7	—	—	no
12	NSI	R5	X4	97 (10%)	6,27	none	LPV/r + TDF+3TC	172 (16%)	2,7	—	—	no
13	NSI	R5	R5	354 (23%)	4,75	none	f-APV/r+TDF/FTC	290 (26%)	1,7	—	—	no
14	NSI	X4	R5	0	6,46	none	LPV/r + ABC/3TC	43 (2%)	1,7	—	—	no
15	NSI	R5	R5	141(13%)	5,49	none	LPV/r + ABC/3TC	227 (13%)	3,8	—	—	no
16	NSI	R5	R5	101 (14%)	6,57	none	LPV/r + TDF/FTC	531 (23%)	3,1	—	—	no
17	NSI	R5	R5	367 (20%)	4,64	none	LPV/r + TDF/FTC	617 (32%)	1,7	—	—	no
18	NSI	R5	R5	247 (26%)	5,04	none	f-APV/r+ ABC/3TC	541 (30%)	1,7	—	—	no
Antiretroviral experienced patients												
Responders												
19	NSI	R5	X4	161 (12%)	5,77	LPV/r + TDF+3TC	TPV/r+ EFV +TDF+3TC	323 (19%)	1,7	—	—	X4→R5
20	NSI	R5	R5	72 (15%)	6,38	LPV/r + TDF+3TC	TPV/r+TDF+3TC+T-20	273 (21%)	1,7	—	—	R5→X4
21	NSI	R5	R5	264 (30%)	4,80	f-APV/r + DDI+TDF+3TC	TPV/r+TDF+3TC+T-20	459 (34%)	1,7	—	—	R5→X4
22	NSI	R5	R5	562 (29%)	3,27	LPV/r + D4T+3TC	TPV/r+3TC+EFV	603 (32%)	1,7	—	—	no
23	NSI	R5	R5	194 (11%)	4,39	EFV + TDF+3TC	TPV/r+TDF+3TC+T-20	324 (18%)	1,7	—	—	no
24	NSI	X4	X4	65 (8%)	4,46	LPV/r + TDF+3TC	TPV/r+TDF+3TC+T-20	224 (14%)	1,7	—	—	no
25	NSI	R5	R5	468 (18%)	5,11	LPV/r + AZT+3TC	TPV/r+TDF+3TC+T-20	367 (20%)	1,7	—	—	no
26	NSI	X4	X4	235 (7%)	5,00	LPV/r+3TC+TDF	TPV/r+EFV +3TC	295 (8%)	1,7	—	—	no
27	NSI	X4	X4	311 (17%)	4,36	SQV/r + AZT/3TC	TPV/r+TDF+3TC+T-20	470 (16%)	1,7	—	—	no
Non responders												
28	SI	X4	X4	27 (3%)	4,54	LPV/r + TDF+3TC	TPV/r+ EFV +TDF/FTC	22 (2%)	5,30	SI→NSI	no	X4→R5
29	SI	R5	R5	30 (5%)	5,84	ATV/r+TDF+3TC +T-20	TPV/r+TDF+3TC+T-20	77 (7%)	5,34	SI→NSI	no	no

30	SI	R5	R5	90 (6%)	2,90	LPV/r+TDF+ ABC +3TC	TPV/r+TDF+ABC+3TC	122 (8%)	4,57	no	no	no
31	NSI	R5	R5	143 (20%)	4,44	f-APV+RTV +ABC + 3TC	TPV/r+ABC/3TC+ T-20	178 (14%)	3,36	no	no	no
32	NSI	R5	R5	170 (25%)	4,71	LPV/r+TDF+3TC	TPV/r+TDF+3TC+T-20	172 (21%)	5,11	no	no	no
33	NSI	X4	R5	123 (5%)	4,79	LPV/r +TDF+3TC	TPV/r+ABC/3TC+EFV	202 (6%)	4,04	no	no	no
34	NSI	R5	R5	145 (7%)	5,27	APV/r+NVF+DDI	TPV/r+TDF+3TC	118 (7%)	5,63	no	no	no
35	NSI	R5	R5	50 (17%)	6,48	ATV/r + DDI + T-20	TPV/r+3TC+T-20	196 (15%)	5,08	no	no	no
36	NSI	X4	R5	27(2%)	5,41	LPV/r +TDF+3TC	TPV/r+TDF+3TC+T-20	97(4%)	4,88	no	X4→R5	no

Legend: Pt: patient; 3TC, lamivudine; AZT, zidovudine; d4T, stavudine; ddl, didanosine; ABC, abacavir; EFV, efavirenz; NVP, nevirapine; ATV, atazanavir; IDV, indinavir; LPV, lopinavir; NFV, nelfinavir; SQV, saquinavir; TPV, tipranavir; f-APV, fosaamprenavir; T-20, enfuvirtide; pVL: plasma viral load; F-up: Follow-up.

Table II. Correlation of baseline patient characteristics with the occurrence of a tropism shift in proviral DNA after one year of antiretroviral therapy.

	No shift	Shift from X4 to R5	<i>p</i> value	Shift from R5 to X4	<i>p</i> value
All patients	n=26	n=6		n=4	
Naives	12	4	0.6	2	1.0
Experienced	14	2		2	
Age (Mean ±SD)		49.0±14.8	0.9	41.2±3.8	0.3
Gender (n)					
male	23	5	0.6	3	0.4
female	3	1		1	
Clinical stage CDC (n)					
stage C	20	3	0.2	3	0.7
others (B+A)	6	3		1	
Risk factor (n)					
sexual	19	4	0.5	3	0.7
parenteral	7	2		1	
Nadir CD4 (cells/mm ³ ±SD)	161.6±150.6	151.3±222.5	0.5	27.2±30.6	0.02
Mean Baseline CD4 (cells/mm ³ ±SD)	172.8±151.7	154.8±220.0	0.4	88.5±120.8	0.2
Mean F _{up} CD4 (cells/mm ³ ±SD)	300.7±166.4	241.5±216.4	0.3	253.5±152.3	0.5
Mean CD4 increase (cells/mm ³ ±SD)	128.0±138.0	86.7±81.9	0.6	165.0±47.7	0.2
Mean Baseline pVL (log ₁₀ cp/ml ±SD)	5.0±0.9	4.8±1.1	0.9	5.4±0.7	0.3
N pts reaching pVL <50cp/ml (n)	15	5	0.2	4	0.1
Time to pVL <50cp/ml (months: mean±SD)	4±3	4±2	0.9	2±1	0.1
Use of enfuvirtide (n)	17	1	0.4	2	0.5
Primary pol resistance associated mutations (n)					
- RT	3±3	3±4	0.5	4±5	0.5
- PR	4±4	3±4	0.4	4±4	0.7
Previous ARV exposure (years: mean±SD)	7±7	5±8	0.7	7±8	0.9

Legend: N: number; SD: standard deviation; CDC: Center for Diseases Control; F-up: Follow-up; pVL: plasma viral load; RT: reverse transcriptase; PR: protease; ARV: antiretroviral therapy

Figure 1. Serial V3 loop sequences of patients changing their V3 genotype during HAART.

Pt	Source	V3 aminoacidic sequence																														n	NC	PSSM											
		HxB2	C	T	R	P	N	N	N	T	R	K	R	I	R	I	Q	R	G	P	G	R	A	F	V	T	I	_	_	G	K				_	I	G	N	M	R	Q	A	H	C	
1.	RNA bl	.	.	.	.	.	.	.	.	.	.	S	.	H	M	_	_	_	.	.	.	.	.	.	Y	A	T	_	_	.	D	I	.	.	D	I	.	K	.	.	.	35	6	R5	
	DNA bl	.	.	.	.	S	.	.	.	.	.	G	.	.	.	_	_	_	.	.	.	.	.	.	Y	A	T	_	_	E	R	I	V	.	I	.	.	.	.	35	7	X4			
	DNA fu	.	.	.	.	S	.	.	.	.	.	G	V	H	.	_	_	_	.	.	.	.	.	.	L	F	R	_	_	T	S	I	T	.	D	I	.	.	.	.	34	7	R5		
2.	RNA bl	.	.	.	.	.	.	.	.	.	.	S	.	P/S/R/C			.	.	.	.	.	.	.	.	F	A	T	_	_	G	D/G	I	.	.	D	I	.	.	.	.	35	4-6	R5		
	DNA bl	.	.	.	.	.	.	.	.	.	.	G	.	.	.	_	_	_	.	.	.	.	.	.	I	A	T	_	_	E	K	I	.	.	I	.	.	.	.	35	7	X4			
	DNA fu	.	.	.	.	.	.	.	.	.	.	S	.	S	.	_	_	_	.	.	.	.	.	.	V/F	A	T	_	_	G	D	I	.	.	D	I	.	.	Y	.	35	3	R5		
3.	RNA bl	.	.	.	.	.	.	.	.	.	.	S	.	T	.	_	_	_	.	.	.	.	.	V	.	D	A	T	_	_	D	D	I	.	.	D	I	.	K/Q	.	Y	.	34	2-3	X4/R5
	DNA bl	.	.	.	.	.	.	.	.	.	.	S	.	T	.	_	_	_	.	.	.	.	.	V	.	D	A	T	_	_	D	I	.	.	D	I	.	K	.	Y	.	34	3	X4	
	DNA fu	.	.	.	.	.	.	.	.	I	R	S	.	H	M	_	_	_	.	.	.	.	.	.	T	.	T	_	_	.	D	I	.	.	D	I	.	K	.	.	35	5	R5		
4.	RNA bl	.	.	.	.	.	.	.	.	.	.	S	.	P	M	_	_	_	.	.	.	.	.	.	Y	.	T	_	_	.	Q	I	.	.	D	I	.	.	.	F	.	35	4	R5	
	DNA bl	.	.	.	.	.	.	.	.	.	.	R/S	.	P	I/L	_	_	_	.	.	.	.	.	A/V	.	Y	.	T	_	.	Q	I	.	.	D	I	.	.	.	F	.	35	4-5	X4/R5	
	DNA fu	.	.	.	.	.	.	.	.	.	.	S	I/V	P	M	_	_	_	.	.	.	.	.	.	Y	.	T	_	.	Q	I	.	.	D	I	.	.	.	F	.	35	4	R5		
5.	RNA bl	.	.	.	.	.	.	.	.	.	R	S	.	H	.	_	_	_	.	.	.	.	.	.	Y	_	_	_	G	T	D	V	.	.	D	I	.	.	Y	.	34	4	R5		
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	DNA fu	.	T	.	.	.	.	.	.	.	.	G	.	.	.	_	_	_	.	.	.	.	.	R	F	Y	A	-	T	.	K	I	.	.	D	I	.	.	.	.	35	7	X4		
19.	RNA bl	.	.	.	.	S	.	.	.	.	.	S	.	P	M	_	_	_	.	.	.	.	.	.	Y	.	T	_	_	.	D	.	.	.	I	.	.	.	.	35	5	R5			
	DNA bl	.	.	.	.	.	.	.	.	.	.	R	.	T	L	_	_	_	.	.	.	.	.	V	Y	Y	.	T	_	.	Q	I	.	.	.	I	.	R	.	35	7	X4			
	DNA fu	.	.	.	.	N/S	.	.	.	.	.	S	.	H/P	I/M	_	_	_	.	.	.	.	.	K/R	.	Y	.	T	_	.	D	I	.	.	.	I	.	.	.	35	5-6	R5			
20.	RNA bl	.	.	.	.	.	.	.	.	.	K/R	G	.	N/H/S/R			.	.	.	.	.	.	.	A/S	.	N/Y/I/F	A	R/T	_	E/G/D	R	I	.	.	D	I	.	.	.	.	35	6-7	R5		
	DNA bl	.	.	.	.	.	.	.	.	.	.	S	.	P	.	_	_	_	.	.	.	.	.	.	Y	.	T	_	_	.	D	I	.	.	.	I	.	.	.	35	5	R5			
	DNA fu	.	.	.	.	.	.	.	.	R	.	G	.	S	.	_	_	_	.	.	.	.	.	S	.	I	A	R	_	D	R	I	.	.	D	I	.	.	.	.	35	6	X4		
21.	RNA bl	.	.	R/T	.	N/S	.	.	.	.	.	G	.	H	.	_	_	_	.	.	.	.	.	V	Y	.	A	-	T	Q	I	.	.	D	I	.	.	.	.	35	5-6	R5			
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	DNA fu	.	.	.	.	N/S	.	.	.	.	.	G	.	H	.	_	_	_	.	.	.	.	.	V	Y	.	A	-	T	K/Q	I	.	.	D	I	.	.	.	.	35	6-7	X4/R5			
28.	RNA bl	.	.	.	.	Y	K/D	K	.	K	R	R	.	Q	.	_	_	_	.	.	.	.	.	.	V	-	-	-	-	T	D	I	Q	.	D	L	.	L	.	Y	.	33	4-6	X4	
	RNA fu	.	.	.	.	Y	E	K	.	K	R	R	.	K	.	_	_	_	.	.	.	.	.	.	V	-	-	-	-	T	D	I	K	.	D	L	.	L	.	Y	.	33	6	X4	
	DNA bl	.	.	.	.	Y	K	K	.	K	R	R	.	Q	.	_	_	_	.	.	.	.	.	.	V	-	-	-	-	T	D	I	Q	.	D	L	.	L	.	Y	.	33	6	X4	
	DNA fu	.	.	.	.	.	.	.	.	.	K/R	S	.	H	.	_	_	_	.	.	.	.	.	.	Y	T/A	T	-	-	T	E/D	I	I	.	D	I	.	Q/R	.	.	.	35	5	R5	

Legend: Pt=Patient; n: number of aminoacids (V3 sequence length); NC=net charge; PSSM (Position-Specific Scoring Matrix).
Columns corresponding to positions 11 and 25 of the env V3 sequence are highlighted.

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Title page

Co-receptor switch during HAART is independent of virological success

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Running head: Co-receptor switch during HAART

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ABSTRACT

The influence of antiretroviral therapy on co-receptor tropism remains controversial. To verify if co-receptor tropism shift was affected by HAART, the evolution of proviral DNA V3 genotype after 12 months of a new antiretroviral regimen was compared between responder and non-responder patients.

Baseline blood samples were collected from 36 patients infected with HIV-1 subtype-B (18 naïve and 18 experienced) for virus isolation and *env* V3 genotyping from plasma HIV-1 RNA and PBMC DNA. DNA V3 genotyping was repeated after 12 months from initiating HAART. WebPSSM was used for categorizing V3 sequences into X4 or R5; for analysis purposes, dual/mixed viruses were considered as X4.

From the 10 (28%) patients changing their proviral DNA V3 genotype during therapy, six shifted from R5-to-X4 and four from X4-to-R5. The lack of reaching virological suppression was not associated with an X4-to-R5 ($p=0.25$) or R5-to-X4 ($p=0.14$) shift; time-to-viral suppression and CD4 increase were similar in both groups. No association was found between tropism shift and patient baseline characteristics including age, sex, CDC stage, CD4 count, viral load, exposure and length of previous HAART, enfuvirtide use in the new regimen, number of reverse-transcriptase and protease resistance-associated mutations. Conversely, CD4 nadir was correlated to emergence of X4 virus in proviral DNA (mean 27.2 ± 30.6 in R5-to-X4 shifting patients vs 161.6 ± 150.6 in non-shifting patients, $p=0.02$).

The occurrence of a tropism shift in both directions was independent of HAART use, irrespective of its efficacy. The CD4 count nadir was the only baseline characteristic able to predict an R5-to-X4 viral shift.

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KEY WORDS: HIV-1, biological phenotype, *env* V3 loop, co-receptor

For Peer Review

INTRODUCTION

The third variable (V3) region of the HIV-1 gp120 envelope is known to be responsible for viral tropism. Specific amino acid variations in the V3 loop, especially the distribution of charged amino acids, have been correlated with the type of co-receptor used by HIV-1 [de Jong et al., 1992; Fouchier et al., 1995]. Together with CD4, two different chemokine receptors (CXCR4 and CCR5) are employed mainly by the virus for entry into cells and therefore, HIV-1 variants can be distinguished into X4 and R5 variants [Deng et al., 1996]; dual/tropic strains and/or mixture of the two variants have also been described (D/M or X4/R5 viruses). The use of co-receptor can be tested by adopting different cell lines, each expressing one of the two co-receptors; one commercial recombinant phenotype assay (Trofile™ Monogram Biosciences, San Francisco, CA, USA) [Whitcomb et al., 2007] and some in-house methods are available. This classification has replaced the previous distinction of viral isolates on MT-2 cells as syncytium inducing (SI) and non-syncytium inducing (NSI) [Schuitemaker H et al., 1991] since MT-2 cells are exclusively permissive to infection by SI viruses because they only express CXCR4 on their surface [Berger et al., 1999]. The correlation between these different methods for determining co-receptor tropism (V3 genotype, MT-2 isolation and phenotype) is still not clear and discrepancies have been demonstrated between these approaches [Low et al., 2007], even if a substantial correlation has been found by more recent studies [Garrido et al., 2008, Raymond et al., 2008; Poveda et al., 2009; Chueca et al., 2009]. Easier and less expensive methods for determining the co-receptor tropism of HIV are required, as they are no longer used exclusively for research but have entered into clinical practice, given the development of new chemokine-co-receptor inhibitors.

Many aspects, however, are still unresolved. Firstly, it is known that variants able to utilize CXCR4, which target preferentially naïve and resting CD4⁺ T-lymphocytes and have an

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2 73 higher replication rate than R5 variants, emerge in approximately 40-50% of infected
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4 74 persons over the course of disease [Berger et al., 1999]. However, whether these X4
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6 75 isolates arise as a consequence of the loss of CD4⁺ cells, or precede it (in which case they
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8 76 might be involved causally in CD4⁺ cell depletion), is still debated. Moreover, it is not
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10 77 known if the presence of X4 viruses, which affect immune progenitor cells at the thymus
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12 78 level, might cause an impairment of CD4 reconstitution even during HAART [Moore et al.,
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14 79 2004; Delobel et al., 2006].
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18 80 Previous studies based on HIV-1 isolation in MT-2 cells have correlated the presence of
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20 81 SI variants to a worse clinical outcome in terms of CD4 decrease, viral load, disease stage
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22 82 and mortality [Berger et al., 1999; Connor, HO 1994; Karlsson et al., 1994; Maas et al.,
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24 83 2000; de Roda et al., 1997]. Also studies based on V3 genotype [Brumme et al., 2004] or
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26 84 phenotype [Daar et al., 2007, Waters et al., 2008] agree that the use of the X4 co-receptor
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28 85 is associated with a more rapid HIV-1 disease progression, while results regarding the
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30 86 response to HAART are equivocal; some authors [Brumme et al., 2004] have found that
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32 87 response to antiretroviral therapy is diminished in patients harbouring a X4 rather than a
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34 88 R5 variant, whereas other studies exclude the direct effect of co-receptor tropism on
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36 89 therapy response [Waters et al., 2008].
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42 90 On the other hand, the influence of HAART on tropism shift is unclear. It has been
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44 91 hypothesized that antiretroviral therapy might exert a different selective pressure on X4 or
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46 92 R5 variants in the viral population. Some studies reported a preferential suppression of X4
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48 93 viruses with HAART [Philpott et al., 2001; Equils et al., 2000, Skrabal et al., 2003, Galan
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50 94 et al., 2004] while other studies suggested a predominant R5 to X4 switch in cell reservoirs
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52 95 with effective therapy [Johnston et al., 2003, Delobel et al., 2005]. More recently, a greater
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54 96 overall prevalence of X4 viruses was observed in experienced-patient cohorts [Melby et
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56 97 al., 2006; Wilkin et al., 2007; Hunt et al., 2006; Coakley et al., 2006] when compared to
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naïve patients [Brumme et al., 2005; Moyle et al., 2005 ; Demarest et al., 2004; Coakley et al., 2006; Poveda et al., 2007].

The use of co-receptors is now mandatory for patients who are candidates to receive an anti-CCR5 inhibitor, but viral tropism shifts between the time of testing and start of therapy have been described [Fätkenheuer et al., 2008]. Whether some patient characteristics might be predictive of tropism shift is still unknown; their identification would certainly help to better define therapeutic strategies including virus entry inhibitors. Therefore, the aim of the present study was to compare the V3 genotype at baseline and after initiating a new antiretroviral therapy in two groups of patients (naïve and experienced) according to their response to a new HAART regimen.

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2 108 **PATIENTS AND METHODS**

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4 109 **Patients**

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7 110 A total of 36 patients infected with HIV were enrolled in the study, including 18
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9 111 antiretroviral-naïve patients and 18 patients failing HAART, whose clinical, virological and
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11 112 immunological characteristics are shown in Table I.

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14 113 Naïve patients were enrolled consecutively from newly diagnosed patients requiring
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16 114 antiretroviral therapy; all were diagnosed with advanced stage of disease (CD4 cell count
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18 115 <350 cells/mm³) with the exception of patients 4, 11, 13 and 17 who, despite of an high
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20 116 absolute number of CD4 cells/mm³, required treatment with HAART due to presence of
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22 117 clinical symptoms.

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26 118 Antiretroviral-experienced patients were enrolled from those participating in an expanded
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28 119 access program for a new antiretroviral drug (tipranavir) and, according to the protocol
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30 120 inclusion criteria, had been treated previously with all three antiretroviral classes including
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32 121 at least two PI-based regimens. Blood samples from all patients were collected at baseline
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34 122 (from a few days up to two weeks before starting or changing the antiretroviral regimen) for
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36 123 virus isolation and genotyping of the *env* V3 region on plasma RNA and PBMC DNA.

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40 124 Patients were followed monthly for virological and immunological parameters and clinical
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42 125 evolution; for patients achieving virological success (HIV-RNA <50 cp/ml), V3 genotyping
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44 126 was determined on PBMC DNA 12 months after initiating HAART while for patients who
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46 127 did not response to therapy and remained viremic, virus isolation and genotyping of the
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48 128 *env* V3 region were determined on both plasma RNA and PBMC DNA 12 months after
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50 129 initiating HAART.

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METHODS

Phylogenetic analysis of *pol* and *env* gene sequences (neighbor-joining method using Kimura two-parameter distances) from these patients was performed to confirm that all enrolled patients were infected with a subtype B HIV-1.

Plasma HIV-RNA was assessed using a NASBA real time PCR (Nuclisens Easy-Q HIV-1, Biomérieux, Borel, France) with a lower detection limit of 50 copies/ml.

Peripheral blood CD4⁺T lymphocytes were quantified by flow cytometry using a commercially-available monoclonal antibodies (Beckman Coulter, Inc., Fullerton, CA).

HIV-1 isolation

Peripheral blood mononuclear cells (PBMCs) were obtained by centrifuging whole blood on a Ficoll-Hypaque density gradient. A total of 5×10^6 PBMCs were co-cultivated with 5×10^6 phytoemagglutinin (PHA)-stimulated PBMCs from healthy seronegative donors in 5 ml of RPMI 1640 supplemented with 10% of Human IL-2, 10% of fetal bovine serum, glutamine (2nM), 100 IU/ml penicillin and 100 µg/ml streptomycin. On the seventh day of culture and then twice weekly, supernatants were collected, stored at -80 °C and replaced with fresh medium. Cultures, placed in a humidified chamber at 37 °C with 5% CO₂ air, were maintained for 27 days. Viral growth on cell culture was demonstrated by p24 antigen production using a commercially available kit (VIDAS HIV P24 II, Biomérieux, Marcy l'Etoile, France). A culture was considered positive if the concentration of p24 was higher than 5.0 pg/ml. Positive supernatants were used for infecting 10⁶ MT-2 cells; cultures were maintained for 2 weeks, examined for syncytia twice a week and supernatant aliquots were stored at -80 °C. Viral cultures with syncytium formation were defined as having a SI biological phenotype, whereas cultures without syncytium formation were defined as having a NSI biological phenotype.

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2 155 **V3 loop genotyping**
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4 156 Using standard methods, HIV-1 proviral DNA and RNA were extracted from the
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7 157 PBMC and plasma samples of all 36 patients, respectively, at time of virus isolation.

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9 158 After cDNA was obtained from HIV-RNA by retrotranscription, the cDNA and proviral DNA
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11 159 from PBMCs were amplified by a nested PCR using outer primers MK603 (forward) 5'
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13 CAGAAAAATTGTGGGTCACAGTCTATTATGGGGTACCT 3' (HXB2 nt 6316-6353) and
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15 CD4R (reverse) 5' TATAATTCACCTTCTCCAATTGTCC 3' (HXB2 nt 7675-7652), and
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17 inner primers: SQV2F1 (forward) 5' ACACATGCCTGTGTACCCACAGA 3' (HXB2 nt
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19 162 6435-6457) and V3O2R (reverse) 5' ATTACAGTAGAAAAATTCCCCTCCAC 3' (HXB2 nt
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21 163 7382-7357). A PCR product of 947 base pairs was obtained, including almost the entire
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23 164 gp120 from codon 43 of C1 to codon 2 of V4.
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28 166 If no product was amplified, alternative first and second-round forward primers were
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30 167 employed: V3O2F (forward) 5' AATGTCAGYACAGTACAATGTACAC 3' (HXB2 nt 6945-
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32 6969) V3O2R (reverse) 5' ATTACAGTAGAAAAATTCCCCTCCAC 3' (HXB2 nt 7382-7357)
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34 as outer primers, and V3I2F (forward) 5' TAAATGGCAGTYTAGCAGAAGAAG 3' (HXB2
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36 nt 7006-7029) V3I2R (reverse) 5' ACAATYTCTGGGTCCCCTCCTGAGGA 3' (HXB2 nt
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38 7039-7314) as inner primers, thus obtaining a smaller PCR product of length 333 bp,
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40 171 including the V3 loop from codon 65 of C2 to codon 42 of C3.
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44 173 PCR products were then sequenced in both 5' and 3' directions using the BigDye dye
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46 terminator cycle sequencing kit (Applied Biosystems, Foster City, CA) with primers V3O2F,
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48 V3O2R, V3I2F, V3I2R for gp120, and V3I2F and V3I2R for V3, and run on an ABI 310
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50 automated sequencer.
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54 177 A multiple alignment of patient sequences (GenBank accession numbers DQ984217-
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56 178 DQ984276; DQ841560-DQ841561; DQ841564-DQ841567; DQ841569; FJ798215-
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58 798302) with a reference alignment of the V3 region provided by the HIV sequence
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60 database of Los Alamos was obtained using Clustal W2. Only the alignment with the HxB2
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reference sequence has been reported; as sequence length ranged from 33 to 37 amino acids (aa), gaps were inserted for sequences of a length < 37 aa.

The Position-Specific Scoring Matrix PSSM method, freely available on the web (<http://ubik.microbiol.washington.edu/computing/pssm/>), was used arbitrarily for categorizing V3 sequences into X4 or R5 phenotype, by means of an X4R5 matrix calculated by comparing patient sequences to sequences of known co-receptor phenotype. This comparison produces a score: the higher the score, the more closely the sequence resembles those of known X4 viruses [Jensen et al., 2003]. When nucleotide mixtures were observed, nucleic acid sequence from each patient were translated into amino-acidic sequences containing all possible combinations; a X4 genotype was assigned arbitrarily if at least 25% of all possible combinations from a given patient resulted in a X4 virus according to PSSM.

Statistical analysis

For the purpose of this study, dual/mixed virus (X4/R5) were considered as X4.

Descriptive statistics were calculated for the demographic, clinical and virological-immunological characteristics of all cases. Mean and standard deviation (SD) are presented for normally distributed variables.

The Kruskal-Wallis test was utilized to compare quantitative variables, and Pearson's Chi-square test (Fisher exact test where appropriate) was used for categorical variables. The concordance between genotypic and phenotypic testing was analyzed by means of Kappa-statistics. Data analysis was performed with Epi Info™ Version 3.4.3.

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Results

Viro-immunological response to HAART

Viro-immunological characteristics of patients in the study are shown in Table I. Naïve patients (pt 1-18) presented a mean CD4 count of 146.6 ± 171.2 and a mean plasma viral load $\log_{10}$ 5.1 ± 0.9 at baseline. In 17/18 patients, a TAMs-sparing regimen based on abacavir or tenofovir plus lamivudine or emtricitabine was chosen, in combination with efavirenz in 2/18 patients and with a boosted PI in 16/18 patients; in one patient, T20 was also included in the first PI-based regimen. A total of 15/18 patients achieved virological success (83%); the median time to virological suppression was 3 months (range: 1 to 10). In three patients (pt 12, 15 and 16), plasma viral load was still detectable after 12 months, most likely due to a low adherence rate. In all patients, independent of viral suppression, a CD4 increase from baseline was observed, with a mean gain of CD4+ cells/mm³ equal to 173.4 ± 139.1 .

Antiretroviral-experienced patients (pt 19 to 36) had a mean CD4 count of 174.3 ± 149.7 and a pVL of 4.9 ± 0.9 at time of failure; they were all NNRTI-experienced, and had been treated previously with a mean number of 5 ± 1 NRTIs and 4 ± 1 PIs, respectively; 7/18 patients were also enfuvirtide-experienced. The mean length of previous antiretroviral therapy was 14 ± 4 years. All patients started a new antiretroviral regimen including boosted tipranavir; 11/18 patients were also treated with enfuvirtide. Patients 19-27 achieved virological success after a median time of three months (range 1 to 3); in this group of patients the mean CD4 increase after one year was 111.8 ± 96.9 cells. Patients 28 to 36 did not respond to therapy and remained viremic; after a mean 12 month follow-up, the mean pVL was 4.81 ± 0.7 ; nevertheless, a mean CD4 count increase of 42.1 ± 52.1 was also obtained for these non-responder patients.

Co-receptor characteristics at baseline.

The biological phenotype determined with MT-2 cell isolation (SI/NSI) and the predicted co-receptor use according to the V3 genotype for each patient are also shown in Table I.

In 27/36 (75%) patients, a concordant baseline V3 genotype was observed in plasma and PBMCs (Kappa agreement = 0.39). In the plasma compartment, a X4 virus was noted in 9/36 (25%) patients, three of whom were naïve (17%) and six were experienced (33%).

The presence of a X4 variant did not correlate to any of the investigated clinical variables (gender, age, risk factor, CDC stage, baseline pVL). The CD4 nadir and the absolute number of CD4 cells at baseline, however, were lower in X4 patients, without a statistical significant difference between the two groups.

Also in the proviral DNA compartment where a total of 11/36 (30.5%) patients (six of whom were naïve and five were experienced patients) harboured a X4 genotype, no association was found with any clinical variable, including baseline CD4 cells/mm³ and pVL.

SI isolates were cultured in five patients, only one of whom (pt 28) showed a complete agreement with the presence of a X4 genotype in both RNA and DNA. The overall concordance between MT-2 isolation and predicted co-receptor use based on RNA and DNA genotype was 69% (K=0.10) and 72% (K=0.23), respectively. An association between the presence of an SI isolate and a lower number of nadir (36.8 ± 34.1 cells/mm³ in patients carrying SI viruses vs 162.4 cells/mm³ ± 164.5 in those carrying NSI viruses, $p=0.03$) and baseline CD4 cell count (40.8 ± 30.8 cells/mm³ vs 179.7 ± 163.2 cells/mm³ in the two groups, respectively, $p=0.05$) was described, while no correlation was found between SI phenotype and baseline pVL (4.8 ± 1.2 log₁₀ cp/ml vs 5.0 ± 0.9 log₁₀ cp/ml, respectively, $p=0.9$).

In the group of experienced patients, the length of the previous treatment with antiretrovirals was not associated with the presence of SI variants at MT-2 isolation ($p=0.9$) nor with X4 variants either in plasma RNA ($p=0.2$) or in the proviral DNA ($p=0.9$).

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Association between baseline co-receptor usage and viro-immunological outcome

The probability of achieving virological suppression after 12 months of therapy was not influenced by the presence of a X4 variant in plasma RNA (OR=1.23; 0.26 - 5,98 95% IC; p= 0.56) or PBMC DNA (OR=3. 00; 0.53-16,90 95% IC; p= 0.19), nor by the presence of SI isolates at baseline (OR=0.27; 0.04 – 1.92 95% IC; p=0.19). In addition, the mean increase in CD4+ cells was similar for patients with a SI versus a NSI isolate (p=0.30) and for patients with X4 versus a R5 variant in plasma (p=0.47) or in PBMCs (p=0.97); when considering the absolute number of CD4 cells/mm³ at follow-up, the CD4+ count was significantly lower in patients with an SI isolate at baseline (p<0.001).

V3 genotype shift and response to HAART

When analyzing the proviral DNA compartment, a total of 10/36 (28%) patients (6/18 naïve and 4/18 experienced patients) switched their V3 genotype during the first 12 months of the new antiretroviral therapy (Table I); the *env* V3 sequences of these patients at baseline and at one-year follow-up are reported in Figure 1. In particular, in the naïve patient group, four subjects showed a shift from a X4 (pt 1-3) or X4/R5 (pt 4) to a R5 genotype after starting HAART. All these patients were virological responders and experienced a mean increase of 91±80 CD4/mm³ during the course of therapy. Conversely, in two cases (pt 5 and 6), the emergence of a X4 genotype was described after 12 months of therapy, together with an undetectable viral load: also in this case an increase in the CD4 cell count was obtained (mean 132±49 CD4 cells/mm³). No changes in V3 genotype were observed for the three naïve patients (pt 12, 15 and 16) who did not reach viral suppression after one year of therapy, even if they all showed a pVL decrease in viral load (-3,57, -1,69, -3.47 log₁₀ cp/ml, respectively).

In the group of experienced patients, a X4 to R5 shift was described both in virological responders (pt 19) and in non-responders (pt 28), while two virological responder patients (pt 20 and 21) showed a reversion from a R5 to a X4 genotype.

Among non-responders, virus isolation and RNA genotyping were also performed; two cases of reversion from a SI to a NSI phenotype were observed with HAART which was confirmed in only one case by DNA genotyping. Lastly, in one case a change from an X4 to an R5 genotype was described only in RNA, but not in DNA or by virus isolation.

Overall, the lack of achieving virological suppression was not associated with a shift in proviral DNA from X4 to R5 (OR=3.67; 0.37-35.9 95% IC, $p=0.25$) or from R5 to X4 (RR=1.3, 1.00-1.60 95% IC, $p=0.14$); time-to-viral-suppression was also similar in the two patient groups ($p=0.9$ and 0.1 , respectively). No differences in terms of CD4 increase in both groups were observed ($p=0.6$ and $p=0.2$, respectively)(Table II).

Correlation between baseline patient characteristics and occurrence of a tropism shift

The following baseline variables did not demonstrate any statistically significant association with a tropism shift in both directions: age, gender, CDC stage of infection, risk factor for HIV-1, previous exposure to antiretroviral therapy, length of previous HAART for the group of experienced patients, baseline CD4 count and pVL (Table II). On the contrary, a lower CD4 cell count nadir was associated with a greater probability of a R5 to X4 shift in the proviral compartment (mean CD4 nadir 27.2 ± 30.6 in shifting patients vs 161.6 ± 150.6 in patient not shifting V3 genotype, $p=0.02$).

The presence of a discordant V3 genotype between RNA and DNA at baseline was not associated with a higher probability of shifting during therapy (OR=1.43; 0.28-7.30 95% IC,

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2 305 p=0.49). Also the total number of pol mutations associated with resistance in reverse
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4 306 transcriptase (RT) and protease (PR) was not related to tropism change (Table II).
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7 307 Lastly, the possible effect of including enfuvirtide in the new therapy on genotype shift was
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9 308 investigated, but no association was found between use of enfuvirtide and presence of a
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11 309 shift from a X4 to R5 (OR=0.38, 0.04-3.74 95% IC, p=0.37) or from a R5 to X4 variant
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14 310 (OR=1.89, 0.23-15.74 95% IC, p=0.47) in the proviral compartment.
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Discussion

The *env* gene of HIV-1, which is directly exposed to host immunological pressure, is the most variable viral region. This variability influences the penetration of HIV-1 into different cell types, according to the two main chemokine co-receptor binding variants (X4 and R5) which are known to be correlated to diverse disease progression rates. However, the relationship between the prevalence of X4 and/or R5 strains in the viral *quasispecies* and the use of antiretroviral therapy requires further clarification. In fact, it is not known if HAART might facilitate the emergence of X4 variants, as some reports seem to suggest [Johnston et al., 2003, Delobel et al., 2005], or whether the presence of X4 variants could hamper achievement of virological and immunological success [Brumme et al., 2004].

The aim of our study was to verify if tropism shift in both naïve and experienced patients is affected by HAART; thus, the evolution of the V3 genotype after 12 months of HAART was studied and compared in responder and non-responder patients.

Firstly, a varying frequency of X4 strains in naïve and antiretroviral experienced patient cohorts was described, illustrating a higher amount of X4 strains in experienced [Melby et al., 2006; Wilkin et al., 2007; Hunt et al., 2006; Coakley et al., 2006] than in naïve subjects [Brumme et al., 2005; Moyle et al., 2005; Demarest et al., 2004; Coakley et al., 2006. Poveda et al., 2007]. In fact, an association between the presence of X4 variants and HAART had been noted in our previous study [Saracino et al., 2007]; in the present study, the prevalence of X4 variants at baseline was similar in treated and naïve patients, the majority of whom, however, demonstrated an advanced stage of immunological impairment.

No statistically significant differences were observed in terms of mean CD4 increase nor probability of achieving virological suppression after starting or changing HAART between the two patient groups harboring a X4 or a R5 variant at baseline, respectively, even if patients carrying X4 variants at baseline had a decreased CD4 count, with a consequently

reduced mean absolute number of CD4⁺ cells at follow-up. These results were comparable in naive and experienced patients, but the limited size of the sample did not permit definite conclusions; however, it must be emphasized that in no case was antiretroviral therapy success affected by the presence of a X4 strain in the viral mixture. Previous reports on the HOMER cohort suggested that the presence of X4 variants could be associated with a worse virological response, a finding, however, which was not confirmed by further analyses using co-receptor phenotype testing [Brumme et al., 2005]. Interestingly, and in contrast with earlier reports [Philpott et al., 2001; Equils et al., 2000; Skrabal et al., 2003; Galán et al., 2004; Johnston et al., 2003; Delobel et al., 2005], our study demonstrates that the V3 genotype shift is not influenced by HAART. In fact, a similar number of shifts was observed in both directions (from a X4 to an R5 or from a R5 to an X4 variant) during therapy in patients with homogeneous characteristics at baseline. This is in agreement with more recently published articles [Lwembe et al., 2009; Briz et al., 2008; Souliè et al., 2007] which also found that co-receptor switching is independent of HAART, while it is most likely affected by the rate of disease progression. Also the length of a previous HAART treatment in the group of experienced patients did not seem to influence the detection of a X4 variant at baseline or after therapy change in our population. No associations were found with the number of *pol* resistance associated mutations for NRTI, NNRTIs and PIs [Lehman et al., 2006], nor did the use of enfuvirtide correlate with a tropism change from baseline in our patient group, in contrast to data reported by Melby [Melby et al., 2006].

If the tropism shift is unrelated to HAART, the identification of other predictive factors for tropism change would be of great importance when treating with anti-CCR5 inhibitors; a shift between the testing for coreceptor tropism and the initiation of an anti-CCR5-based therapy is quite common, and might be associated to virological failure [Fätkenheuer et al., 2008]. The majority of baseline features investigated in our cohort, however, did not

demonstrate a sufficient predictive power; in particular, the tropism shift was equally frequent among naïve patients initiating their first regimen and in previously-failed patients after therapy change, and did not seem to be associated with age, gender, clinical stage, modality of acquiring HIV-1 infection, and baseline viro-immunological parameters. Of note, however, the CD4 cell count nadir was associated to a R5-to- X4 tropism shift in our group of patients; if confirmed, this result which is in agreement with several reports which highlight the importance of immune impairment in the emergence of X4 variants, might be helpful for the selection of patients who would benefit from anti-CCR5 therapy and who most likely need to initiate therapy close to the time of screening.

Among the 5/6 patients who switched from X4 to R5 in DNA, five subjects already harbored R5 viruses in the plasma at baseline; therefore, the detection of a R5 strain after a one-year follow-up is probably due to the enrichment of the proviral reservoir by circulating viruses rather than by the *ex-novo* selection of new tropism variants. In some samples, the co-existence of both variants could have been underestimated for viral strains with a frequency below the sensitivity threshold (10-20%) of the current PCR assays. This is an important limitation as these variants could eventually emerge during virological failure with CCR5-antagonist therapy; in the near future, studies based on the new ultra-deep sequencing techniques will permit the evaluation of the impact that these minority variants might have on clinical practice [Paredes and Clotet, 2009]. At present, direct sequencing on plasma and PBMC samples is currently accepted as an appropriate method due to its simplicity and acceptable correlation with clonal analysis [Delobel et al., 2007].

A reduced degree of concordance (Cohen's K statistics 0.39) was observed between the results obtained from the plasma and proviral DNA compartments in paired samples at baseline. In addition, the overall concordance between MT-2 isolation and predicted co-receptor use based on RNA (K=0.10) and DNA genotype (K=0.23) was low. Discrepancies

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2 389 between the two compartments were also noted by other researchers [Verhofstede C et
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4 390 al., 2009]; in a previous study, V3 analysis from plasma samples largely agreed with the
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6 391 corresponding DNA samples but greatly differed from the MT-2 biological phenotype
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8 392 [Saracino et al., 2007]. The differences existing between phenotypic (MT-2 isolation, co-
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10 393 receptor testing) and V3 genotype interpretation methods are not yet completely
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12 394 understood [Low et al., 2007, Garrido et al., 2008; Raymond et al., 2008; Saracino et al.,
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14 395 2007] although the validation of improved algorithms is ongoing [Poveda et al., 2009;
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16 396 Chueca et al., 2009] and must be considered when interpreting the results. However, it
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18 397 must be emphasized that in the current study, a closer association emerged between
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20 398 baseline immunological/clinical characteristics and the biological phenotype when
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22 399 compared to genotyping, thereby suggesting that, in the presence of a mixed viral
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24 400 population including X4R5 variants which are able to use both co-receptors, the finding of
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26 401 an SI isolate by the MT-2 cell assay clearly suggests the presence of a more aggressive
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28 402 virus. In fact, several reports indicate that co-receptor use and real cellular tropism are
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30 403 related, but distinct, whereas tropism reflects the ability of an isolate not only to enter, but
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32 404 also to replicate, in a particular target cell [Goodenow and Collman, 2006]. We
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34 405 acknowledge, however, the purely speculative value of these considerations due to the
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36 406 small sample size and to the lack of a comparison with a tropism assay.
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38 407 In conclusion, in our cohort, the occurrence of a tropism shift both from an X4 to R5 or, on
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40 408 the contrary, from an R5 to a X4 variant was independent of HAART, irrespective of its
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42 409 efficacy. The tropism change in the proviral compartment during antiretroviral therapy
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44 410 could not be predicted from baseline patient features, with the exception of the CD4 cell
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46 411 count nadir which appeared to correlate with the occurrence of R5 to X4 shift.
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