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► **To cite this version:**

Annalisa Saracino, Laura Monno, Donatella C Cibelli, Grazia Punzi, Gaetano Brindicci, et al.. Co-receptor switch during HAART is independent of virological success. *Journal of Medical Virology*, 2009, 81 (12), pp.2036. 10.1002/jmv.21598 . hal-00531822

HAL Id: hal-00531822

<https://hal.science/hal-00531822>

Submitted on 4 Nov 2010

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Co-receptor switch during HAART is independent of virological success

Journal:	<i>Journal of Medical Virology</i>
Manuscript ID:	JMV-09-1289.R1
Wiley - Manuscript type:	Research Article
Date Submitted by the Author:	04-Jun-2009
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Keywords:	HIV-1, biological phenotype, env V3 loop, co-receptor



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

Pt	Baseline patient characteristics						New HAART regimen	Virological and immunological outcome		Co-receptor Shift		
	MT-2 isolate	RNA V3	DNA V3	Baseline CD4	Baseline pVL	Antiretroviral Therapy		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

1													
2	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
3	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
4	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
5	33	NSI	X4	R5	123 (5%)	4,79	LPV/r +TDF+3TC	TPV/r+ABC/3TC+EFV	202 (6%)	4,04	no	no	no
6	34	NSI	R5	R5	145 (7%)	5,27	APV/r+NVF+DDI	TPV/r+TDF+3TC	118 (7%)	5,63	no	no	no
7	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
8	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.

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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 parenteral	19 7	4 2	0.5	3 1	0.7
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 HxB2	V3 aminoacidic sequence																												n	NC	PSSM														
		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	V	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	V	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	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		
	DNA bl	S	.	H	.	-	-	-	-	-	-	-	.	.	Y	-	-	-	A	T	D	V	.	.	D	I	Y	.	.	34	4	R5		
	DNA fu	S	K	T	I	R	R	.	H	.	-	-	-	-	-	-	.	.	Y	A	T	D	V	T	D	V	K	.	D	I	.	.	R	.	Y	.	.	37	7	X4		
6.	RNA bl	.	V	S	.	H	.	-	-	-	-	-	-	-	.	.	Y	-	-	-	-	-	E	I	.	.	D	I	34	5	R5			
	DNA bl	.	V	S	.	H	.	-	-	-	-	-	-	-	.	.	Y	-	-	-	-	-	E	I	.	.	D	I	34	5	R5			
	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			
	DNA bl	S	.	.	.	G	.	H	.	-	-	-	-	-	-	-	.	.	V	Y	.	A	-	-	T	Q	I	.	.	D	I	35	6	R5			
	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	.	Q	.	-	-	-	-	-	-	.	.	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	-	-	-	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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2 1 **Title page**
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30 13 **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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46 **KEY WORDS:** HIV-1, biological phenotype, *env* V3 loop, co-receptor

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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
7
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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40 90 On the other hand, the influence of HAART on tropism shift is unclear. It has been
41
42 91 hypothesized that antiretroviral therapy might exert a different selective pressure on X4 or
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44 92 R5 variants in the viral population. Some studies reported a preferential suppression of X4
45
46 93 viruses with HAART [Philpott et al., 2001; Equils et al., 2000, Skrabal et al., 2003, Galan
47
48 94 et al., 2004] while other studies suggested a predominant R5 to X4 switch in cell reservoirs
49
50 95 with effective therapy [Johnston et al., 2003, Delobel et al., 2005]. More recently, a greater
51
52 96 overall prevalence of X4 viruses was observed in experienced-patient cohorts [Melby et
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54 97 al., 2006; Wilkin et al., 2007; Hunt et al., 2006; Coakley et al., 2006] when compared to
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1
2 98 naïve patients [Brumme et al., 2005; Moyle et al., 2005 ; Demarest et al., 2004; Coakley et
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4 99 al., 2006; Poveda et al., 2007].

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6
7 100 The use of co-receptors is now mandatory for patients who are candidates to receive an
8
9 101 anti-CCR5 inhibitor, but viral tropism shifts between the time of testing and start of therapy
10
11 102 have been described [Fätkenheuer et al., 2008]. Whether some patient characteristics
12
13 103 might be predictive of tropism shift is still unknown; their identification would certainly help
14
15 104 to better define therapeutic strategies including virus entry inhibitors. Therefore, the aim of
16
17 105 the present study was to compare the V3 genotype at baseline and after initiating a new
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19 106 antiretroviral therapy in two groups of patients (naïve and experienced) according to their
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21 107 response to a new HAART regimen.
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2 108 **PATIENTS AND METHODS**

3
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
10
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
21 116 absolute number of CD4 cells/mm³, required treatment with HAART due to presence of
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23 117 clinical symptoms.

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26 118 Antiretroviral-experienced patients were enrolled from those participating in an expanded
27
28 119 access program for a new antiretroviral drug (tipranavir) and, according to the protocol
29
30 120 inclusion criteria, had been treated previously with all three antiretroviral classes including
31
32
33 121 at least two PI-based regimens. Blood samples from all patients were collected at baseline
34
35 122 (from a few days up to two weeks before starting or changing the antiretroviral regimen) for
36
37 123 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
41
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
47 127 did not response to therapy and remained viremic, virus isolation and genotyping of the
48
49 128 *env* V3 region were determined on both plasma RNA and PBMC DNA 12 months after
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51 129 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, Beseind, 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 μ g/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 10^6 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.

1 2 155 **V3 loop genotyping**

3
4 156 Using standard methods, HIV-1 proviral DNA and RNA were extracted from the
5
6
7 157 PBMC and plasma samples of all 36 patients, respectively, at time of virus isolation.

8
9 158 After cDNA was obtained from HIV-RNA by retrotranscription, the cDNA and proviral DNA
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11
12 159 from PBMCs were amplified by a nested PCR using outer primers MK603 (forward) 5'
13
14 160 CAGAAAAATTGTGGGTCACAGTCTATTATGGGGTACCT 3' (HXB2 nt 6316-6353) and
15
16 161 CD4R (reverse) 5' TATAATTCACCTTCTCCAATTGTCC 3' (HXB2 nt 7675-7652), and
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18
19 162 inner primers: SQV2F1 (forward) 5' ACACATGCCTGTGTACCCACAGA 3' (HXB2 nt
20
21 163 6435-6457) and V3O2R (reverse) 5' ATTACAGTAGAAAAATTCCCCTCCAC 3' (HXB2 nt
22
23 164 7382-7357). A PCR product of 947 base pairs was obtained, including almost the entire
24
25
26 165 gp120 from codon 43 of C1 to codon 2 of V4.

27
28 166 If no product was amplified, alternative first and second-round forward primers were
29
30
31 167 employed: V3O2F (forward) 5' AATGTCAGYACAGTACAATGTACAC 3' (HXB2 nt 6945-
32
33 168 6969) V3O2R (reverse) 5' ATTACAGTAGAAAAATTCCCCTCCAC 3' (HXB2 nt 7382-7357)
34
35 169 as outer primers, and V3I2F (forward) 5' TAAATGGCAGTYTAGCAGAAGAAG 3' (HXB2
36
37
38 170 nt 7006-7029) V3I2R (reverse) 5' ACAATYTCTGGGTCCCCTCCTGAGGA 3' (HXB2 nt
39
40 171 7039-7314) as inner primers, thus obtaining a smaller PCR product of length 333 bp,
41
42 172 including the V3 loop from codon 65 of C2 to codon 42 of C3.

43
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45 173 PCR products were then sequenced in both 5' and 3' directions using the BigDye dye
46
47 174 terminator cycle sequencing kit (Applied Biosystems, Foster City, CA) with primers V3O2F,
48
49 175 V3O2R, V3I2F, V3I2R for gp120, and V3I2F and V3I2R for V3, and run on an ABI 310
50
51
52 176 automated sequencer.

53
54 177 A multiple alignment of patient sequences (GenBank accession numbers DQ984217-
55
56 178 DQ984276; DQ841560-DQ841561; DQ841564-DQ841567; DQ841569; FJ798215-
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59 179 798302) with a reference alignment of the V3 region provided by the HIV sequence
60
180 database of Los Alamos was obtained using Clustal W2. Only the alignment with the HxB2

1
2 181 reference sequence has been reported; as sequence length ranged from 33 to 37 amino
3
4 182 acids (aa), gaps were inserted for sequences of a length < 37 aa.
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6

7 183
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9 184 The Position-Specific Scoring Matrix PSSM method, freely available on the web
10
11 185 (<http://ubik.microbiol.washington.edu/computing/pssm/>), was used arbitrarily for
12
13
14 186 categorizing V3 sequences into X4 or R5 phenotype, by means of an X4R5 matrix
15
16 187 calculated by comparing patient sequences to sequences of known co-receptor
17
18 188 phenotype. This comparison produces a score: the higher the score, the more closely the
19
20
21 189 sequence resembles those of known X4 viruses [Jensen et al., 2003]. When nucleotide
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23 190 mixtures were observed, nucleic acid sequence from each patient were translated into
24
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26 191 amino-acidic sequences containing all possible combinations; a X4 genotype was
27
28 192 assigned arbitrarily if at least 25% of all possible combinations from a given patient
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31 193 resulted in a X4 virus according to PSSM.
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33 194 34 35 195 **Statistical analysis**

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37 196 For the purpose of this study, dual/mixed virus (X4/R5) were considered as X4.

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39
40 197 Descriptive statistics were calculated for the demographic, clinical and virological-
41
42 198 immunological characteristics of all cases. Mean and standard deviation (SD) are
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44
45 199 presented for normally distributed variables.

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47 200 The Kruskal-Wallis test was utilized to compare quantitative variables, and Pearson's Chi-
48
49 201 square test (Fisher exact test where appropriate) was used for categorical variables. The
50
51
52 202 concordance between genotypic and phenotypic testing was analyzed by means of
53
54 203 Kappa-statistics. Data analysis was performed with Epi Info™ Version 3.4.3.
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1
2 204 **Results**

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4 205 **Viro-immunological response to HAART**

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7 206 Viro-immunological characteristics of patients in the study are shown in Table I. Naïve
8
9 207 patients (pt 1-18) presented a mean CD4 count of 146.6 ± 171.2 and a mean plasma viral
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11 208 load \log_{10} 5.1 ± 0.9 at baseline. In 17/18 patients, a TAMs-sparing regimen based on
12
13
14 209 abacavir or tenofovir plus lamivudine or emtricitabine was chosen, in combination with
15
16 210 efavirenz in 2/18 patients and with a boosted PI in 16/18 patients; in one patient, T20 was
17
18 211 also included in the first PI-based regimen. A total of 15/18 patients achieved virological
19
20
21 212 success (83%); the median time to virological suppression was 3 months (range: 1 to 10).
22
23 213 In three patients (pt 12, 15 and 16), plasma viral load was still detectable after 12 months,
24
25
26 214 most likely due to a low adherence rate. In all patients, independent of viral suppression, a
27
28 215 CD4 increase from baseline was observed, with a mean gain of CD4+ cells/mm³ equal to
29
30 216 173.4 ± 139.1 .

31
32
33 217 Antiretroviral-experienced patients (pt 19 to 36) had a mean CD4 count of 174.3 ± 149.7
34
35 218 and a pVL of 4.9 ± 0.9 at time of failure; they were all NNRTI-experienced, and had been
36
37 219 treated previously with a mean number of 5 ± 1 NRTIs and 4 ± 1 PIs, respectively; 7/18
38
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40 220 patients were also enfuvirtide-experienced. The mean length of previous antiretroviral
41
42 221 therapy was 14 ± 4 years. All patients started a new antiretroviral regimen including boosted
43
44 222 tipranavir; 11/18 patients were also treated with enfuvirtide. Patients 19-27 achieved
45
46
47 223 virological success after a median time of three months (range 1 to 3); in this group of
48
49 224 patients the mean CD4 increase after one year was 111.8 ± 96.9 cells. Patients 28 to 36 did
50
51
52 225 not respond to therapy and remained viremic; after a mean 12 month follow-up, the mean
53
54 226 pVL was 4.81 ± 0.7 ; nevertheless, a mean CD4 count increase of 42.1 ± 52.1 was also
55
56 227 obtained for these non-responder patients.

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229 **Co-receptor characteristics at baseline.**

1
2 230 The biological phenotype determined with MT-2 cell isolation (SI/NSI) and the predicted
3
4 231 co-receptor use according to the V3 genotype for each patient are also shown in Table I.

6
7 232 In 27/36 (75%) patients, a concordant baseline V3 genotype was observed in plasma and
8
9 233 PBMCs (Kappa agreement = 0.39). In the plasma compartment, a X4 virus was noted in
10
11 234 9/36 (25%) patients, three of whom were naïve (17%) and six were experienced (33%).

13
14 235 The presence of a X4 variant did not correlate to any of the investigated clinical variables
15
16 236 (gender, age, risk factor, CDC stage, baseline pVL). The CD4 nadir and the absolute
17
18 237 number of CD4 cells at baseline, however, were lower in X4 patients, without a statistical
19
20
21 238 significant difference between the two groups.

22
23 239 Also in the proviral DNA compartment where a total of 11/36 (30.5%) patients (six of whom
24
25
26 240 were naïve and five were experienced patients) harboured a X4 genotype, no association
27
28 241 was found with any clinical variable, including baseline CD4 cells/mm³ and pVL.

29
30 242 SI isolates were cultured in five patients, only one of whom (pt 28) showed a complete
31
32
33 243 agreement with the presence of a X4 genotype in both RNA and DNA. The overall
34
35 244 concordance between MT-2 isolation and predicted co-receptor use based on RNA and
36
37 245 DNA genotype was 69% (K=0.10) and 72% (K=0.23), respectively. An association
38
39
40 246 between the presence of an SI isolate and a lower number of nadir (36.8 ± 34.1 cells/mm³
41
42 247 in patients carrying SI viruses vs 162.4 cells/mm³ ± 164.5 in those carrying NSI viruses,
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44
45 248 $p=0.03$) and baseline CD4 cell count (40.8 ± 30.8 cells/mm³ vs 179.7 ± 163.2 cells/mm³ in
46
47 249 the two groups, respectively, $p=0.05$) was described, while no correlation was found
48
49 250 between SI phenotype and baseline pVL (4.8 ± 1.2 log₁₀ cp/ml vs 5.0 ± 0.9 log₁₀ cp/ml,
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51
52 251 respectively, $p=0.9$).

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54 252 In the group of experienced patients, the length of the previous treatment with
55
56 253 antiretrovirals was not associated with the presence of SI variants at MT-2 isolation ($p=0.9$)
57
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59 254 nor with X4 variants either in plasma RNA ($p=0.2$) or in the proviral DNA ($p=0.9$).

60
255

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

257 The probability of achieving virological suppression after 12 months of therapy was not
258 influenced by the presence of a X4 variant in plasma RNA (OR=1.23; 0.26 - 5,98 95% IC;
259 $p= 0.56$) or PBMC DNA (OR=3. 00; 0.53-16,90 95% IC; $p= 0.19$), nor by the presence of
260 SI isolates at baseline (OR=0.27; 0.04 – 1.92 95% IC; $p=0.19$). In addition, the mean
261 increase in CD4+ cells was similar for patients with a SI versus a NSI isolate ($p=0.30$) and
262 for patients with X4 versus a R5 variant in plasma ($p=0.47$) or in PBMCs ($p=0.97$); when
263 considering the absolute number of CD4 cells/mm³ at follow-up, the CD4+ count was
264 significantly lower in patients with an SI isolate at baseline ($p<0.001$).

266 **V3 genotype shift and response to HAART**

267 When analyzing the proviral DNA compartment, a total of 10/36 (28%) patients (6/18 naïve
268 and 4/18 experienced patients) switched their V3 genotype during the first 12 months of
269 the new antiretroviral therapy (Table I); the *env* V3 sequences of these patients at baseline
270 and at one-year follow-up are reported in Figure 1. In particular, in the naïve patient group,
271 four subjects showed a shift from a X4 (pt 1-3) or X4/R5 (pt 4) to a R5 genotype after
272 starting HAART. All these patients were virological responders and experienced a mean
273 increase of 91 ± 80 CD4/mm³ during the course of therapy. Conversely, in two cases (pt 5
274 and 6), the emergence of a X4 genotype was described after 12 months of therapy,
275 together with an undetectable viral load: also in this case an increase in the CD4 cell count
276 was obtained (mean 132 ± 49 CD4 cells/mm³). No changes in V3 genotype were observed
277 for the three naïve patients (pt 12, 15 and 16) who did not reach viral suppression after
278 one year of therapy, even if they all showed a pVL decrease in viral load ($-3,57$, $-1,69$, $-$
279 3.47 log₁₀ cp/ml, respectively).

1
2 280 In the group of experienced patients, a X4 to R5 shift was described both in virological
3
4 281 responders (pt 19) and in non-responders (pt 28), while two virological responder patients
5
6 282 (pt 20 and 21) showed a reversion from a R5 to a X4 genotype.

7
8
9 283 Among non-responders, virus isolation and RNA genotyping were also performed; two
10
11 284 cases of reversion from a SI to a NSI phenotype were observed with HAART which was
12
13 285 confirmed in only one case by DNA genotyping. Lastly, in one case a change from an X4
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15 286 to an R5 genotype was described only in RNA, but not in DNA or by virus isolation.
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21 288 Overall, the lack of achieving virological suppression was not associated with a shift in
22
23 289 proviral DNA from X4 to R5 (OR=3,67; 0,37-35.9 95% IC, p=0.25) or from R5 to X4
24
25 290 (RR=1.3, 1.00-1.60 95% IC, p=0.14); time-to-viral-suppression was also similar in the two
26
27 291 patient groups (p=0.9 and 0.1, respectively). No differences in terms of CD4 increase in
28
29 292 both groups were observed (p=0.6 and p=0.2, respectively)(Table II).
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33 293 34 35 294 **Correlation between baseline patient characteristics and occurrence of a tropism** 36 37 295 **shift**

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40 296 The following baseline variables did not demonstrate any statistically significant
41
42 297 association with a tropism shift in both directions: age, gender, CDC stage of infection, risk
43
44 298 factor for HIV-1, previous exposure to antiretroviral therapy, length of previous HAART for
45
46 299 the group of experienced patients, baseline CD4 count and pVL (Table II). On the contrary,
47
48 300 a lower CD4 cell count nadir was associated with a greater probability of a R5 to X4 shift in
49
50 301 the proviral compartment (mean CD4 nadir 27.2±30.6 in shifting patients vs 161.6±150.6 in
51
52 302 patient not shifting V3 genotype, p=0.02).
53

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56 303 The presence of a discordant V3 genotype between RNA and DNA at baseline was not
57
58 304 associated with a higher probability of shifting during therapy (OR=1.43; 0.28-7.30 95% IC,
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1
2 305 p=0.49). Also the total number of pol mutations associated with resistance in reverse
3
4 306 transcriptase (RT) and protease (PR) was not related to tropism change (Table II).
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6
7 307 Lastly, the possible effect of including enfuvirtide in the new therapy on genotype shift was
8
9 308 investigated, but no association was found between use of enfuvirtide and presence of a
10
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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For Peer Review

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

1
2 337 reduced mean absolute number of CD4+ cells at follow-up. These results were
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4 338 comparable in naive and experienced patients, but the limited size of the sample did not
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6
7 339 permit definite conclusions; however, it must be emphasized that in no case was
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9 340 antiretroviral therapy success affected by the presence of a X4 strain in the viral mixture.
10
11 341 Previous reports on the HOMER cohort suggested that the presence of X4 variants could
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14 342 be associated with a worse virological response, a finding, however, which was not
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16 343 confirmed by further analyses using co-receptor phenotype testing [Brumme et al., 2005].
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18 344 Interestingly, and in contrast with earlier reports [Philpott et al., 2001; Equils et al., 2000;
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21 345 Skrabal et al., 2003; Galán et al., 2004; Johnston et al., 2003; Delobel et al., 2005], our
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23 346 study demonstrates that the V3 genotype shift is not influenced by HAART. In fact, a
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26 347 similar number of shifts was observed in both directions (from a X4 to an R5 or from a R5
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28 348 to an X4 variant) during therapy in patients with homogeneous characteristics at baseline.
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30 349 This is in agreement with more recently published articles [Lwembe et al., 2009; Briz et al.,
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33 350 2008; Souliè et al., 2007] which also found that co-receptor switching is independent of
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35 351 HAART, while it is most likely affected by the rate of disease progression. Also the length
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37 352 of a previous HAART treatment in the group of experienced patients did not seem to
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40 353 influence the detection of a X4 variant at baseline or after therapy change in our
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42 354 population. No associations were found with the number of *pol* resistance associated
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44
45 355 mutations for NRTI, NNRTIs and PIs [Lehman et al., 2006], nor did the use of enfuvirtide
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47 356 correlate with a tropism change from baseline in our patient group, in contrast to data
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49 357 reported by Melby [Melby et al., 2006].
50
51
52 358 If the tropism shift is unrelated to HAART, the identification of other predictive factors for
53
54 359 tropism change would be of great importance when treating with anti-CCR5 inhibitors; a
55
56 360 shift between the testing for coreceptor tropism and the initiation of an anti-CCR5-based
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58
59 361 therapy is quite common, and might be associated to virological failure [Fätkenheuer et al.,
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362 2008]. The majority of baseline features investigated in our cohort, however, did not

1
2 363 demonstrate a sufficient predictive power; in particular, the tropism shift was equally
3
4 364 frequent among naïve patients initiating their first regimen and in previously-failed patients
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6
7 365 after therapy change, and did not seem to be associated with age, gender, clinical stage,
8
9 366 modality of acquiring HIV-1 infection, and baseline viro-immunological parameters. Of
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12 367 note, however, the CD4 cell count nadir was associated to a R5-to- X4 tropism shift in our
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14 368 group of patients; if confirmed, this result which is in agreement with several reports which
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16 369 highlight the importance of immune impairment in the emergence of X4 variants, might be
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19 370 helpful for the selection of patients who would benefit from anti-CCR5 therapy and who
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21 371 most likely need to initiate therapy close to the time of screening.

22
23 372 Among the 5/6 patients who switched from X4 to R5 in DNA, five subjects already
24
25
26 373 harbored R5 viruses in the plasma at baseline; therefore, the detection of a R5 strain after
27
28 374 a one-year follow-up is probably due to the enrichment of the proviral reservoir by
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31 375 circulating viruses rather than by the *ex-novo* selection of new tropism variants. In some
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33 376 samples, the co-existence of both variants could have been underestimated for viral
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35 377 strains with a frequency below the sensitivity threshold (10-20%) of the current PCR
36
37
38 378 assays. This is an important limitation as these variants could eventually emerge during
39
40 379 virological failure with CCR5-antagonist therapy; in the near future, studies based on the
41
42 380 new ultra-deep sequencing techniques will permit the evaluation of the impact that these
43
44
45 381 minority variants might have on clinical practice [Paredes and Clotet, 2009]. At present,
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47 382 direct sequencing on plasma and PBMC samples is currently accepted as an appropriate
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49 383 method due to its simplicity and acceptable correlation with clonal analysis [Delobel et al.,
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51
52 384 2007].

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54 385 A reduced degree of concordance (Cohen's K statistics 0.39) was observed between the
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56 386 results obtained from the plasma and proviral DNA compartments in paired samples at
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58
59 387 baseline. In addition, the overall concordance between MT-2 isolation and predicted co-
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388 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
3
4 390 al., 2009]; in a previous study, V3 analysis from plasma samples largely agreed with the
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6
7 391 corresponding DNA samples but greatly differed from the MT-2 biological phenotype
8
9 392 [Saracino et al., 2007]. The differences existing between phenotypic (MT-2 isolation, co-
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11 393 receptor testing) and V3 genotype interpretation methods are not yet completely
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13
14 394 understood [Low et al., 2007, Garrido et al., 2008; Raymond et al., 2008; Saracino et al.,
15
16 395 2007] although the validation of improved algorithms is ongoing [Poveda et al., 2009;
17
18 396 Chueca et al., 2009] and must be considered when interpreting the results. However, it
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20
21 397 must be emphasized that in the current study, a closer association emerged between
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23 398 baseline immunological/clinical characteristics and the biological phenotype when
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25
26 399 compared to genotyping, thereby suggesting that, in the presence of a mixed viral
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28 400 population including X4R5 variants which are able to use both co-receptors, the finding of
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30 401 an SI isolate by the MT-2 cell assay clearly suggests the presence of a more aggressive
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33 402 virus. In fact, several reports indicate that co-receptor use and real cellular tropism are
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35 403 related, but distinct, whereas tropism reflects the ability of an isolate not only to enter, but
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37
38 404 also to replicate, in a particular target cell [Goodenow and Collman, 2006]. We
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40 405 acknowledge, however, the purely speculative value of these considerations due to the
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42 406 small sample size and to the lack of a comparison with a tropism assay.

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45 407 In conclusion, in our cohort, the occurrence of a tropism shift both from an X4 to R5 or, on
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47 408 the contrary, from an R5 to a X4 variant was independent of HAART, irrespective of its
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49 409 efficacy. The tropism change in the proviral compartment during antiretroviral therapy
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51
52 410 could not be predicted from baseline patient features, with the exception of the CD4 cell
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54 411 count nadir which appeared to correlate with the occurrence of R5 to X4 shift.
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2 412 **ACKNOWLEDGMENTS**
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4 413 We are grateful to Ms. Paulene Butts for the review of the manuscript and Gianfranco
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7 414 Botte for secretarial assistance.
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