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The lowest X4 Geno2Pheno false-positive rate is associated with greater CD4 depletion in HIV-1 infected patients

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Abstract

Through this study we evaluated whether the HIV-1 tropism determined by genotypic analysis correlates with HIV-1 markers, such as CD4 cell count and plasma HIV-RNA. The analysis was performed on 1221 HIV-1 B-subtype infected patients with an available V3 sequence (all maraviroc naive). Of them, 532 were antiretroviral therapy (ART) naive and 689 ART experienced. Tropism determination was performed by using the geno2pheno (co-receptor) algorithm set at a false-positive rate (FPR) of 10% and 2%. Potential associations of FPR with CD4 cell count and viraemia were evaluated. Association of V3 mutations with genotypic-determined tropism was also evaluated according to different FPR ranges. About 26% of patients (either ART naive or ART experienced) were infected by X4-tropic viruses (using the classical 10% FPR cut-off). However, a significantly lower proportion of ART-naive patients had FPR ≤ 2% in comparison with ART-experienced patients (4.9% vs. 12.6%, respectively, p <0.001). The risk of advanced HIV-1 infection (with CD4 cell count ≤ 200 cells/mm^3) was significantly greater in X4-infected patients, either ART-naive (OR (95% CI)), 4.2 (1.8–9.2); p 0.0006) or ART-experienced (2.3 (1.4–3.6); p 0.0003), with FPR set at 2% (but not at 10%). This finding was confirmed by multivariable logistic analysis. No relationship was found between viraemia and FPR ≤2%. Some X4-related mutations were significantly associated with FPR ≤2% (ART-naive patients, S11R, Y21V, G24K and G24R, p ≤0.001; ART-experienced patients, Y7K, S11R, H13Y, p ≤0.002). In conclusion, these findings show that within the context of genotypically-assessed CXCR4 tropism, FPR ≤2% defines (far better than 10%-FPR) a viral population associated with low CD4 rank, with potentially greater cytopathic effect, and with more advanced disease.

Keywords: Advanced HIV-1 infection, CD4 cell count, false-positive rate, HIV-genotypic tropism, V3 mutations, viraemia
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Introduction

Human immunodeficiency virus type 1 (HIV-1) entry into host cells is a multistep process that requires sequential interactions of the envelope glycoprotein gp120, first with the CD4 receptor and then with one of the family chemokine receptors, mainly CCR5 or CXCR4. The V3 loop in HIV-1 gp120 has been shown to be critical for co-receptor binding [1], and HIV-1 strains can be phenotypically classified according to virus ability to use the CCR5 (R5) and/or CXCR4 (X4) co-receptor. Pure R5-tropic and pure X4-tropic viruses can use only the CCR5 and CXCR4 co-receptors to enter target cells, respectively, while a dual-tropic virus can use both co-receptors. In a dual/mixed-tropic viral population, the use of both co-receptors can be due to the presence of dual-tropic species, to a mixture of pure R5-tropic and X4-tropic species, or both [2].

HIV-1 co-receptor usage is of central pathological and clinical importance. Indeed, it has been shown that the use

of the CXCR4 co-receptor is generally seen in more advanced stages of disease, and has been associated with an increased severity of HIV disease, higher viral load, and a decreased CD4 cell count [3–6].

Blocking the interaction between gp120 and the viral co-receptors on the host cell has been achieved by using small molecules able to bind CXCR4 or CCR5 co-receptors: chemokine co-receptors have thus become a new target for antiretroviral therapy (ART).

AMD3100 (plerixafor) and AMD3465 are CXCR4 coreceptor antagonists able to inhibit HIV-1 [7]. Although CXCR4 antagonists are the first inhibitors discovered, they are not in clinics for HIV-1 treatment because they showed poor efficacy and no oral bioavailability [7,8]. In contrast, maraviroc, the first antagonist of CCR5 co-receptor, has been approved for treating HIV-1 disease only in patients infected by CCR5-tropic viruses and is currently used in clinical practice. On this basis, the determination of HIV-1 tropism is mandatory before the prescription of this CCR5 antagonist [9–11]. In previous years, co-receptor usage determination was assessed using the phenotypic assay Trofile (Monogram Biosciences, South San Francisco, CA, USA) [12]. Today, genotypic tropism testing is commonly carried out in clinical practice by using the genetic information contained in the sequence of HIV-1 gp120 V3-loop through web-based bioinformatic interpretation tools [1,13,14]. Among the available algorithms used for genotypic tropism determination, geno2pheno(co-receptor) (G2P) is currently the most used and promising tool, due to its good concordance with phenotypic results [15–17; 18th Conference on Retroviruses and Opportunistic Infections, abstract 667]. The result of the interpretation of this system is given as a percentage score, the false-positive rate (FPR), ranging from 0% to 100%, that positively predicts the use of the CCR5 co-receptor. Even though European guidelines advise use of the G2P interpretation system with the FPR set at 10% [11], there is evidence that indicates that G2P can provide reliable discrimination between R5 and X4 sequences also when FPR is set between 5% and 10% [47th Meeting of Infectious Diseases Society of America, abstract 297; 17th Conference on Retroviruses and Opportunistic Infections, abstract 92]. Furthermore, recent studies that were carried out by using ultradeep 454-pyrosequencing (UDPS) revealed new important information about the relevance of very low FPR. First of all, by UDPS the highest prevalence of X4 species (range 35–98%) of the entire viral population was detected in patients with a FPR <2% obtained by population sequencing [18th Conference on Retroviruses and Opportunistic Infections, abstract 667]. In addition, by longitudinal analysis, UDPS showed that only CXCR4-using HIV populations with an extremely low FPR (<5%) were selected by maraviroc in patients who started an anti-CCR5 therapy with X4 dual/mixed viruses at baseline [International Workshop on HIV and Hepatitis Virus Drug Resistance and Curative Strategies, abstract 76].

These results suggest that the characterization of HIV species with distinct values of FPR may provide additional information regarding tropism characteristics of the viral populations present in plasma, but also regarding the biological characteristics of the virus (replication capacity, cytopathic effect, etc.).

Therefore, the aim of this study is to evaluate whether a genotypic analysis of co-receptor tropism correlates with HIV-related markers such as CD4 cell count and plasma HIV-RNA in ART-naive and ART-experienced patients.

Materials and Methods

Patients

The study included 1573 HIV-1-infected patients followed in different hospitals in central Italy. In order to reduce the data contamination (and the potential misinterpretation from a clinical perspective) induced by the natural variability of HIV subtypes, only subtype-B HIV-1 infected patients were analysed. For this reason, 352 patients carrying the non-B subtype were excluded from the study, which focused on 1221 (all subtype B) infected patients.

The HIV-1 gp120 V3 region was available for all 1221 patients. The majority of V3 sequences were performed for research purposes (about 97%), while the remaining 3% were screened before initiating treatment with maraviroc. At the time of genotypic test, all patients were naive to maraviroc. In particular, 532 patients were ART naive, while 689 were ART-experienced patients (about 30% in therapy-interruption for any reason, all others in therapeutic failure).

V3 sequencing

HIV-1 gp120 V3 loop sequencing was performed on plasma samples by using a well-validated research-use protocol, based on commercially available RNA-extraction (QIAamp RNA Viral Mini kit, Qiagen, Valencia, CA, USA), reverse-transcription and amplification (SuperScript™ One-Step RT-PCR for Long Templates; Invitrogen) and genotyping (BigDye terminator v.3.1 cycle sequencing kit; Applied Biosystems, Foster City, CA, USA) kits, as previously described [17]. Amplified Gp120 V3 products were full-length sequenced in sense and antisense orientations by an automated sequencer (ABI 3130) by using four different overlapping sequence-specific primers to ensure the coverage of the V3 sequence by at least two sequence segments [17].

Genotypic subtyping

HIV-1 subtype was determined by using phylogenetic analysis on HIV-1 V3 sequences. Briefly, the sequences were aligned with HIV-1 reference sequences of all subtypes (http://www.hiv.lanl.gov). The alignment was edited using the BioEdit program version 7.0.5.3. Phylogenetic trees were estimated using the PAUP* package [18]. The transversion model (GTR + I + G) of nucleotide substitution was chosen using Modeltest v.3.7 implemented in PAUP* [19], and then manually modified to optimize parameter settings for each dataset. Maximum likelihood trees were inferred from selected models using tree bisection-reconnection (TBR) branch swapping.

Genotypic prediction of viral tropism

HIV-1 co-receptor usage was determined from the V3 nucleotide sequence by using the G2P algorithm available at the following website: http://coreceptor.bioinf.mpi-inf.mpg.de/ [13]. G2P was set at FPR of 10%, thus patients with FPR ≤10% were considered infected with X4-tropic viruses according to guidelines [11]. Moreover, based on the recent observations on the enrichment in the X4 viral population and the loss of maraviroc activity related to FPR ≤2% [18th Conference on Retroviruses and Opportunistic Infections, abstract 667; 47th Meeting of Infectious Diseases Society of America, abstract 297], the X4-infected patients were explored more deeply by further categorization into two different subgroups: patients with FPR ≤2% and patients with FPR ranging from 2% to 10%.

Statistical analysis

All analyses were performed using the statistical software package SPSS (version 17.0) for Windows (SPSS Inc., Chicago, IL, USA). ART-naive and ART-experienced patients were analysed as two separate populations.

Genotypic prediction of viral tropism

Differences in the prevalence of the two 'X4-tropic' subgroups (FPR≤2% and FPR 2–10%) among ART-naive patients and ART-experienced patients were tested for by Fisher's exact test; p values <0.05 were considered statistically significant.

Evaluation of relationship between genotypic tropism prediction and immuno-virological parameters. The prevalence of X4-infected patients was calculated and compared according to several ranges of CD4 cell count (≤200, 200–350, 350–500, >500 cells/mm^3) and HIV-RNA (<2, 2–3, 3–4, 4–5, 5–5.69, >5.69 $\log_{10}$ copies/ml). Differences in the proportion of X4- or R5-infected patients within different ranges of CD4 cell count and viral load were evaluated by both chi-square test for trend and Fisher exact test.

Particular attention was paid to patients with advanced HIV-1 infection (having CD4 cell count ≤200 cells/mm^3). The risk of having advanced HIV infection was evaluated among patients having different FPR ranges: (i) X4 (FPR ≤ 10%) vs. R5 (FPR > 10%); (ii) X4 (FPR 2–10%) vs. R5 (FPR > 10%); (iii) X4 (FPR ≤ 2%) vs. X4 (FPR 2–10%); (iv) X4 (FPR ≤ 2%) vs. X4 + R5 (FPR > 2%). This risk was calculated as odds ratio (OR) with 95% confidence (CI) interval by 2 × 2 contingency tables.

In order to evaluate the role of genotypic tropism in disease progression, multivariable logistic regression analyses were also performed both in ART-naive and ART-experienced patients by adjusting for the following variables: plasma HIV-RNA at the time of V3 genotyping, age, sex and risk transmission factor. For ART-experienced patients the following variables were also considered: number of ART regimens, years of ART treatment at the V3 genotyping and therapy status (treatment vs. interrupted). The analyses were performed in a subset of ART-naive patients (n = 332) and ART-experienced (n = 288) patients, for whom all the confounding variables were available. Analysis of the missing data was performed to evaluate if the subsets were representative of the full set population; p values <0.05 were considered statistically significant.

V3 mutation prevalence and association with different FPR ranges. In order to assess the association of V3 mutations with genotypic-determined tropism, the prevalence of mutations was calculated and compared in different FPR ranges (≤2%, 2–10%, >10%). Statistically significant differences in the mutation frequency between the different groups were calculated by using Fisher's exact test. The Benjamini–Hochberg method was used to correct for multiple testing at a false discovery rate of 0.05 [20]. All mutations that were found at the 35 V3 positions with an overall prevalence ≥1% were evaluated.

Results

Patients' characteristics

Patients' characteristics are summarized in Table 1. As expected, the median plasma HIV-RNA and CD4 cell count were higher in ART-naive patients than in ART-experienced patients (plasma HIV RNA, 4.7 (4.2–5.3) vs. 4.3 (3.5–4.9) $\log_{10}$ copies/ml, p <0.001, by Mann Whitney *U*-test; CD4-cell count, 331 (201–448) vs. 282 (138–422) cells/mm^3, p <0.001).

TABLE 1. Patients' characteristics

Characteristics	ART-naive patients (*n* = 532)	ART-experienced patients (*n* = 689)
Age (years), median[a]	38	45
Gender, male %	87.1	71.6
Risk transmission factor, %[b]		
Heterosexual	24.4	28.8
Homosexual	52.4	20.4
Sexual	12.2	8.5
IDU	10.4	39.2
Other (iatrogenic or perinatal transmission)	0.6	3.1
CDC stage, %[c]	A (56.3) B (31.1) C (12.6)	A (19.0) B (31.6) C (49.4)
Viraemia (log_{10} copies/ml), median (IQR)[d]	4.7 (4.2–5.3)	4.3 (3.5–4.9)
CD4 cell count, median (IQR) (cells/mm^3)[d]	331 (201–448)	282 (138–422)
Pts with CD4 cell count ≤200 cells/mm^3, %[d]	23.5	33.9
Therapy protocol at V3 sequencing, *n*	–	Treated: 425 Treatment interruption: 186 Unknown: 78
Previous treatment, median (IQR)[e]		
Number of regimens		4 (2–7)
Number of ARV drugs		8 (4–10)
Experienced drug class, %[e]		
NRTI		100
NNRTI		67.8
PI		85.2
FI		7.0
INI		8.8
Years of ART treatment[e], median (IQR)	-	10 (5–14)

ART, antiretroviral therapy; ARV, antiretroviral; FI, fusion inhibitors; IDU, injection drug user; INI, integrase inhibitors; NRTI, nucleoside/nucleotide reverse transcriptase inhibitors; NNRTI, non-NRTI; pts, patients; PI, protease inhibitors.
[a]Age was available for 497 ART-naive patients and 580 ART-experienced patients.
[b]Risk transmission factors were available for 393 ART-naive patients and 520 ART-experienced patients.
[c]CDC stage at the moment of V3 sequencing was available for 206 ART-naive patients and 256 ART-experienced patients.
[d]Available values at the moment of V3 sequencing.
[e]Complete therapeutic history was available for 406 ART-experienced patients.

Prevalence of patients infected with X4-tropic viruses

Overall, 314 out of 1221 (25.7%) patients showed X4-using viruses at genotypic tropism testing (FPR set at 10%). No significant differences in the prevalence of X4-using viruses was observed between ART-naive and ART-experienced patients (25.0% vs. 26.2%, p 0.644, Fisher's exact test) (Fig. 1). However, when the analysis was performed using 2% as FPR cut-off, a lower proportion of ART-naive patients had FPR ≤2% in comparison with ART-experienced patients (4.9% vs. 12.6%, respectively, p <0.001) (Fig. 1). Within ART-experienced patients, a lower prevalence of X4 tropic virus was found among patients in treatment-interruption in comparison with those who were treated (FPR set at 10%, 20.4% vs. 28.4, p 0.040; FPR set at 2%, 7.5 vs. 14.3, p 0.014).

Evaluation of relationship between genotypic tropism prediction and immuno-virological parameters

According to different CD4 cell count ranges (≤200, 200–350, 350–500, >500 cells/mm^3), significant differences in the proportion of X4-infected patients with FPR ≤2% were observed, both in ART-naive (11.2% vs. 4.7%, 2.7% and 0.8%, respectively, p <0.001 by chi-square for trend) and ART-experienced patients (19.2% vs. 8.6%, 8.9%, and 10.2% respectively, p 0.003) (Fig. 2a,b). It is noteworthy that, within ART-experienced patients, a different trend in the proportion of tropism groups was observed for CD4 cell count range 200–350 mm^3 (Fig. 2b). This atypical trend can be explained by the different tropism prevalence found between treated patients (FPR (%) ≤2, 9.9%; FPR 2–10, 14%; FPR > 10, 76%) and those who interrupted therapy (FPR (%) ≤ 2, 5.7%; FPR 2–10, 3.8%; FPR > 10, 90.6%) (p 0.063).

However, we did not observe differences in tropism prevalence in the stratum of patients with CD4 ≤200 cells/mm^3; of interest is that we observed no significant differences when the same analysis was performed for patients with FPR 2–10% along different CD4 cell count ranges. Therefore, the difference in genotypic tropism found in patients with CD4 ≤200/mm^3, was mostly accounted for by FPR rank ≤2%.

When we considered different FPR ranges according to HIV-RNA ranges, no significant differences in the proportion of patients infected by X4-tropic viruses were observed, both in ART-naive and ART-experienced patients (data not shown). Therefore, FPR ≤2% significantly correlates with immunological status, but not with viral load.

Risk of having advanced HIV-1 infection according to X4-tropism

In ART-naive patients, by setting FPR at 2%, the risk of having advanced HIV-1 infection (CD4 ≤200/mm^3) was significantly higher in patients with FPR ≤2% vs. FPR > 2% (OR (CI 95%), 4.2 (1.8–9.2), p 0.0006), while this risk was not significant when FPR was set at 10% (Fig. 3).

The role of X4 tropism as a significant independent predictor of advanced HIV-1 infection was confirmed, with FPR set at 2%, also by multivariable logistic regression (OR (95% CI), 3.4 (1.0–11.643), p 0.047).

In ART-experienced patients, FPR set at 2% was also associated with a significantly higher risk of having advanced HIV-1 infection compared with patients infected by viruses with FPR >2% (OR (95% CI), 2.3 (1.4–3.6), p 0.0003). In contrast to the ART-naive population, this finding was significant also for FPR set at 10% (Fig. 3). By multivariable logistic regression analysis (after adjusting for all the confounders indicated in the Materials and Methods section, including therapy interruption), tropism was confirmed as an independent predictor of advanced HIV-1 infection by setting FPR at both 2% (OR (CI 95%), 6.0 (2.4–15.0), p <0.0001) and 10% (3.0 (1.6–2.7), p 0.001).

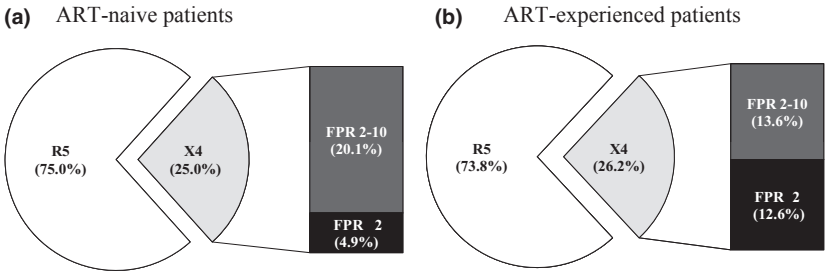

FIG. 1. Proportion of patients infected with X4- and R5-tropic viruses. (Panel a) Antiretroviral therapy (ART)-naive patients. (Panel b) ART-experienced patients. The proportions of R5-infected (FPR>10%, in white) and X4-infected (FPR≤10%, grey lines) patients are indicated in the pie plots. Exploded bars represent the stratification of X4-infected patients according to 2% FPR-X4 ranges (FPR 2–10%, in dark grey; FPR≤2%, in black).

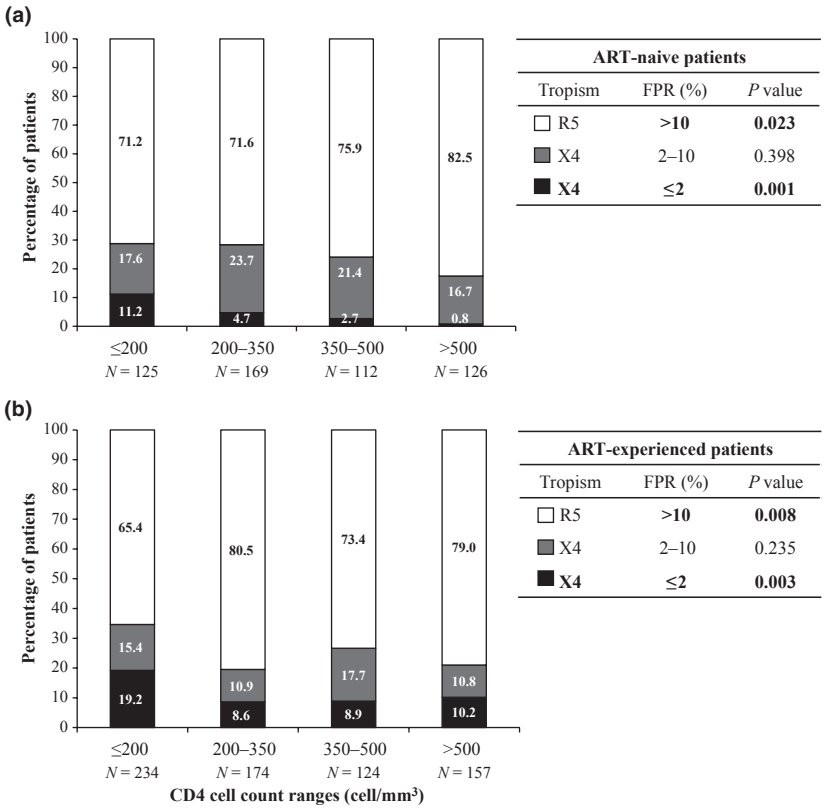

FIG. 2. Proportion of X4- and R5-infected patients according to different CD4 cell count ranges. (Panel a) Proportion of antiretroviral therapy (ART)-naive patients stratified for different CD4 cell count ranges. (Panel b) Proportion of ART-experienced patients stratified for different CD4 cell count ranges. Bar plots indicate the proportion of X4-infected patients with FPR ≤2% (in black) or FPR 2–10% (in grey), and the proportion of R5-infected patients (in white), all of them stratified for different CD4 cell count ranges. Tables inserted in the figure indicate the p values obtained by chi-square test for trend used to calculate potential differences in the proportion of patients for the following groups: (i) patients with FPR ≤2% vs. those with FPR >2%; (ii) patients with FPR 2–10% vs. those with FPR >10%, (iii) patients with FPR ≤2% vs. those with FPR >2%. In bold are reported p values considered statistically significant (<0.05).

All these findings reinforce the relationship between very low FPR and advanced HIV-1 infection, though with different evidence (and potentially mechanisms) in ART-naive and ART-experienced patients.

V3 mutations associated with different FPR ranges

Table 2 shows the prevalence of mutations according to FPR ranges (≤2%, 2–10%, >10%). Among 85 V3 mutations found with prevalence ≥1% in our cohort of ART-naive patients,

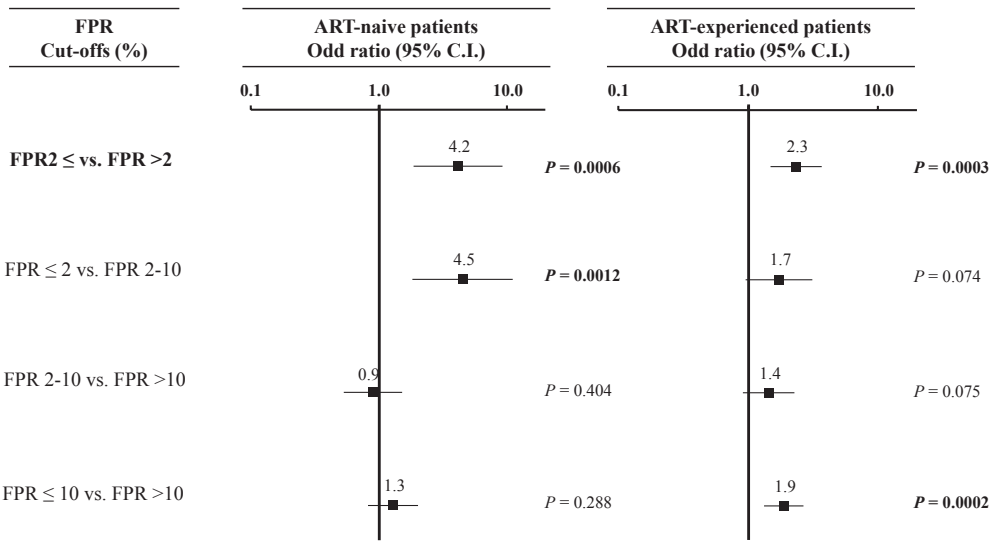

FIG. 3. Risk of having CD4 cell count ≤200 cells/mm^3 by false-positive rate ranges in antiretroviral therapy (ART)-experienced and ART-naive patients. Forest plots represent the odds ratios (with 95% confidence interval) of having CD4 cell count ≤200 cells mm^3 calculated comparing several categories of patients having different FPR ranges. On the left panel are ART-naive patients; on the right panel are ART-experienced patients; p values were obtained by Fisher's exact test and were considered statistically significant at a threshold of 0.05.

only five were significantly associated with FPR ≤2%. Four of them (S11R, Y21V, G24K, G24R) also remained strongly associated with FPR ≤2% after multiple comparisons. It is noteworthy that the prevalence of S11R mutation was strongly associated with FPR ≤2% (p <0.001). Similarly, the mutations Y21V, G24K and G24R were highly present in patients with FPR ≤2% and nearly absent in patients with FPR >2% (p ≤0.001).

Among 91 V3 mutations found with prevalence ≥1% in ART-experienced patients, only eight mutations were significantly associated with FPR ≤2%. Six of them (N7K, N7Y, S11R, H13Y, H13S and R18S) remained strongly associated with FPR ≤2% after multiple comparisons. In particular, mutations N7K and H13Y were present in patients with FPR ≤2% and nearly absent in other patients (p ≤0.002). As in naive patients, it is noteworthy that the prevalence of the S11R mutation was strongly associated with FPR ≤2% and it was completely absent in patients with FPR >10%.

Discussion

The present study, which was carried out with a large cohort of ART-naive and ART-experienced patients, shows that a genotypic analysis of co-receptor tropism correlates with CD4 cell count (but not with viral load). The lowest X4 FPR was associated with greater CD4 depletion in HIV-1 infected patients. So far, few studies (with a relatively small number of patients) have highlighted a similar type of association [6,16; 3rd Italian Conference on AIDS and Retroviruses, abstract SC16; International Workshop on HIV and Hepatitis Virus Drug Resistance and Curative Strategies, abstract 89].

In the present study, about one-quarter of patients (either ART naive or ART experienced) were carrying predominant X4-tropic viruses (using the classical 10% FPR cut-off). A higher proportion of X4-tropic virus was also found in patients with a relatively high CD4 cell count (as shown in Fig. 2). This result was surprising because the appearance of the X4 virus is commonly considered as typical of advanced stages of the disease, thus suggesting that the pathogenetic mechanisms of progression of HIV infection are more complex than thought up to now. So far, co-receptor tropism testing remains mandatory in all patients planning to start therapy with CCR5 antagonists, independently of their CD4 number and stage of the disease.

Genotypic tropism analysis was also performed using 2% as FPR cut-off, a category that better defines pure X4 virus, insensitive to CCR5 antagonists, and it was compared with the classical FPR set at 10% [47th meeting of Infectious Diseases Society of America, abstract 297; International Workshop on HIV and Hepatitis Virus Drug Resistance and Curative Strategies, abstract 76]. A lower proportion of ART-naive patients carrying predominant viruses with FPR ≤2% compared with ART-experienced patients (4.9% vs. 12.6%, respectively) was found. Viruses with FPR ≤2% were nearly absent (0.8%) in ART-naive patients with CD4 > 500 cells/mm^3, compared with almost 20% prevalence in the same

TABLE 2. Prevalence of V3 mutations associated with different FPR ranges in ART-naive and ART-experienced patients

		Prevalence according with FPR ranges N (%)					
FPR ranges		**≤2%**	**2–10%**		**>10%**		**Overall**
ART-naive patients		***N* = 25**	***N* = 108**	**p Value[a]**	***N* = 399**	**p Value[b]**	***N* = 532**
Position	Mutations						
2	T2M	0 (0.0)	5 (4.6)	0.583	1 (0.3)	**0.002**	6 (1.1)
9	R9S	3 (12.0)	8 (7.4)	0.432	6 (1.5)	**0.003**	17 (3.2)
11	S11R	23 (92.0)	7 (6.5)	**<0.001**	0 (0.0)	**<0.001**	30 (5.6)
12	I12L	0 (0.0)	6 (5.6)	0.594	0 (0.0)	**<0.001**	6 (1.1)
13	H13G	1 (4.0)	6 (5.6)	1.000	2 (0.5)	**0.002**	9 (1.7)
14	I14L	2 (8.0)	25 (23.1)	0.105	24 (6.0)	**<0.001**	51 (9.6)
14	I14V	1 (4.0)	5 (4.6)	1.000	2 (0.5)	**0.006**	8 (1.5)
16	P16G	1 (4.0)	4 (3.7)	1.000	1 (0.3)	**0.008**	6 (1.1)
19	A19V	8 (32.0)	14 (13.0)	0.034	17 (4.3)	**0.002**	39 (7.3)
20	F20V	1 (4.0)	6 (5.6)	1.000	1 (0.3)	**<0.001**	8 (1.5)
20	F20Y	4 (16.0)	9 (8.3)	0.265	6 (1.5)	**0.001**	19 (3.6)
21	Y21V	5 (20.0)	0 (0.0)	**<0.001**	8 (2.0)	0.212	13 (2.4)
21	Y21H	1 (4.0)	9 (8.3)	0.687	2 (0.5)	**<0.001**	12 (2.3)
22	T22A	12 (48.0)	58 (53.7)	0.661	286 (71.7)	**0.001**	356 (66.9)
23	T23A	2 (8.0)	8 (7.4)	1.000	8 (2.0)	**0.009**	18 (3.4)
24	G24R	6 (24.0)	2 (1.9)	**0.001**	1 (0.3)	0.116	9 (1.7)
24	G24K	4 (16.0)	0 (0.0)	**0.001**	2 (0.5)	1.000	6 (1.1)
24	G24E	6 (24.0)	18 (16.7)	0.395	10 (2.5)	**<0.001**	34 (6.4)
25	E25K	3 (12.0)	16 (14.8)	1.000	11 (2.8)	**<0.001**	30 (5.6)
25	E25R	3 (12.0)	11 (10.2)	0.727	8 (2.0)	**<0.001**	22 (4.1)
32	Q32R	2 (8.0)	13 (12.0)	0.736	13 (3.3)	**0.001**	28 (5.3)
ART-experienced patients		***N* = 85**	***N* = 96**	**p Value[a]**	***N* = 508**	**p Value[b]**	***N* = 689**
7	N7K	8 (9.4)	0 (0.0)	**0.002**	0 (0.0)	1.000	8 (1.2)
7	N7Y	12 (14.1)	2 (2.1)	**0.004**	0 (0.0)	0.025	14 (2.0)
9	R9S	7 (8.2)	8 (8.3)	1.000	3 (0.6)	**<0.001**	18 (2.6)
11	S11R	54 (63.5)	12 (12.5)	**<0.001**	0 (0.0)	**<0.001**	66 (9.6)
12	I12V	8 (9.4)	12 (12.5)	0.636	19 (3.7)	**0.001**	39 (5.7)
13	H13Y	13 (15.3)	0 (0.0)	**<0.001**	2 (0.4)	1.000	15 (2.2)
13	H13R	9 (10.6)	9 (9.4)	0.808	13 (2.6)	**<0.001**	31 (4.5)
13	H13S	20 (23.5)	7 (7.3)	**0.003**	30 (5.9)	0.641	57 (8.3)
14	I14V	8 (9.4)	8 (8.3)	0.800	7 (1.4)	**<0.001**	23 (3.3)
14	I14L	14 (16.5)	14 (14.6)	0.837	32 (6.3)	**0.010**	60 (8.7)
15	G15A	0 (0.0)	0 (0.0)	1.000	29 (5.7)	**0.008**	29 (4.2)
18	R18S	1 (1.2)	18 (18.8)	**<0.001**	49 (9.6)	0.013	68 (9.9)
19	A19V	25 (29.4)	11 (11.5)	0.003	18 (3.5)	**0.003**	54 (7.8)
20	F20I	3 (3.5)	9 (9.4)	0.142	15 (3.0)	**0.007**	27 (3.9)
22	T22A	37 (43.5)	53 (55.2)	0.137	363 (71.5)	**0.003**	453 (65.7)
23	T23A	6 (7.1)	15 (15.6)	0.103	22 (4.3)	**<0.001**	43 (6.2)
24	G24D	1 (1.2)	9 (9.4)	0.020	4 (0.8)	**<0.001**	14 (2.0)
24	G24R	10 (11.8)	6 (6.3)	0.294	4 (0.8)	**0.002**	20 (2.9)
24	G24E	15 (17.6)	20 (20.8)	0.707	11 (2.2)	**<0.001**	46 (6.7)
25	E25T	2 (2.4)	5 (5.2)	0.450	1 (0.2)	**<0.001**	8 (1.2)
25	E25R	17 (20.0)	16 (16.7)	0.570	7 (1.4)	**<0.001**	40 (5.8)
25	E25K	18 (21.2)	22 (22.9)	0.858	13 (2.6)	**<0.001**	53 (7.7)
25	E25D	12 (14.1)	17 (17.7)	0.548	243 (47.8)	**<0.001**	272 (39.5)
32	Q32R	16 (18.8)	14 (14.6)	0.549	18 (3.5)	**<0.001**	48 (7.0)

ART, antiretroviral therapy; FPR, false positive range.
p Values significant at a false discovery rate of 0.05 following correction for multiple comparison are shown in boldface.
[a]p Value for comparison between FPR ≤2% vs. FPR2–10% groups.
[b]p Value for comparison between FPR 2–10% vs. FPR >10% groups.

category of patients, if the FPR threshold is set at 10% (instead of 2%). This discrepancy suggests that today the use of FPR needs refinement to better and more uniformly identify those patients that, carrying a predominant pure X4 virus, have very low/no chances of taking advantage of CCR5 antagonists.

The setting of FPR at ≤2% shows a tight relationship between this parameter and CD4 ≤200 cells/mm^3, which was not found in ART-naive patients with FPR set at 10%, that, in ART-naive patients, could not be found with FPR set at 10%. This suggests that viral strains with FPR ≤2% might be associated with a more cytopathic effect. A recent study, performed in our laboratory on 54 HIV-1 primary isolates, supports this hypothesis [21]. Indeed, viral isolates with FPRs <2% were associated with an extensive prevalence of X4-using viruses, with a syncytium-inducing phenotype, a marked cytopathic effect and loss of activity of CCR5-antagonist maraviroc *in vitro*. On the contrary, clinical isolates with FPR ranging from 2% to 10% (as well as nearly all isolates with FPR >10%) were unable to induce syncytium formation and most of them were still sensitive to maraviroc [21]. Of interest, is that our data do not show any significant relationship between tropism and viraemia. In the same *in vitro* experiments reported above [21], viral production was similar in CD4-T cells infected by clinical isolates with FPR<2%

compared with the others. All together, our data support the hypothesis that FPR values that are particularly low are related to the cytopathic effect of the virus, and, in turn, to loss of CD4 cell count, but not viral load.

Genotypic analysis confirmed the difference between viruses with FPR ≤2% compared with 2–10%. Indeed, X4-related mutation S11R and some other X4-related mutations [13,22] were found in ART-naive patients (Y21V, G24K and G24R) or ART-treated patients (Y7K, H13Y) as strongly associated with FPR ≤2% (but not with FPR 2–10%), thus suggesting their contribution to the characterization of 'pure-X4-tropic viruses'.

All these data together show that FPR ranges between 2% and 10% represent a grey area, not necessarily representing pure X4-tropic viruses, and not necessarily associated with low CD4 cell count (and therefore with advanced stages of disease).

This study may have some limitations. First of all, genotypic tropism was determined by the analysis of only the V3 sequences. In this regard, even if a single specific amino acid change in the V3 loop can switch viral co-receptor usage [23–25], it is known that other residues outside of the V3 loop within gp120 and gp41 could be relevant for viral co-receptor usage [26–28]. Our cohort includes only subtype B viruses. Therefore, the results obtained in this study cannot be applied to other cohorts containing non-B viruses. Another potential limitation is that this study is cross-sectional; therefore it is not designed to define whether X4 viruses with FPR ≤2% are a cause or consequence of having low CD4 cell count. Regarding this, recent studies suggest that the appearance of the X4-tropic virus is more a consequence of depletion of the immune system than the cause [29]. Finally, it would be interesting to evaluate the relationship between the duration of HIV-1 infection and the genotypic tropism. However, analysing patients from clinical practice, it was not possible to evaluate this issue, because the diagnosis is frequently made after a time of infection that cannot be quantified.

In conclusion, very low FPR defines patients carrying a viral population significantly associated with low CD4 rank, and thus with a greater risk of advanced disease. All these findings together suggest that low FPR (≤2%) may better identify those patients whose virus is insensitive to CCR5-inhibitors, and can be a surrogate marker of a compromised immune system.

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Transparency Declaration

The authors have no conflicts of interest to declare.

Appendix

Members of the OSCAR (Optimizing the Susceptibility to CCR5 Antagonists Response) Study group

The complete list of centres and members participating in the OSCAR programme is as follows. 'San Raffaele' Hospital (Milan): Adriano Lazzarin, Massimo Clementi, Silvia Nozza, Filippo Canducci, Enzo Boeri. 'L. Sacco' Hospital (Milan): Giuliano Rizzardini, Massimo Galli, Valeria Micheli. 'S. Paolo' Hospital (Milan): Antonella D'Arminio Monforte. Busto Arsizio Hospital (Busto Arsizio [MI]): Tiziana Quirino. 'S. Gerardo' Hospital, (Monza [MI]): Andrea Gori. Ospedali Riuniti (Bergamo): Franco Maggiolo, Anna Paola Callegaro. IRCCS Policlinico S. Matteo (Pavia): Renato Maserati, Fausto Baldanti, Stefania Paolucci. University of Turin (Turin): Giovanni Di Perri, Valeria Ghisetti, Tiziano Allice. Policlinico 'S. Orsola-Malpighi' (Bologna): Marco Borderi, Maria Carla Re, Isabella Bon. 'San Martino' Hosptial (Genova): Claudio Viscoli, Antonio Di Biagio, Bianca Bruzzone. Policlinico of Modena (Modena): Cristina Mussini, William Gennari, Monica Pecorari. Marche Politechnic University Medical School (Ancona): Andrea Giacometti, Alessia Monachetti, Patrizia Bagnarelli.

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References

1. Jensen MA, Li FS, van't Wout AB *et al.* Improved coreceptor usage prediction and genotypic monitoring of R5-to-X4 transition by motif analysis of human immunodeficiency virus type 1 env V3 loop sequences. *J Virol* 2003; 77: 13376–13388.
2. Berger EA, Doms RW, Fenyö EM *et al.* A new classification for HIV-1. *Nature* 1998; 39: 240.
3. Feinberg MB, McCune JM, Miedema F *et al.* HIV tropism and CD4 + T-cell depletion. *Nat Med* 2002; 8: 537.
4. Waters L, Mandalia S, Randell P *et al.* The impact of HIV tropism on decreases in CD4 cell count, clinical progression, and subsequent response to a first antiretroviral therapy regimen. *Clin Infect Dis* 2008; 46: 1617–1623.
5. Goetz MB, Leduc R, Kostman JR *et al.* Relationship between HIV coreceptor tropism and disease progression in persons with untreated chronic HIV infection. *J Acquir Immune Defic Syndr* 2009; 50: 259–266.
6. Raymond S, Delobel P, Mavigner M *et al.* CXCR4-using viruses in plasma and peripheral blood mononuclear cells during primary HIV-1 infection and impact on disease progression. *AIDS* 2010; 24: 2305–2312.
7. De Clercq E. The AMD3100 story: the path to the discovery of a stem cell mobilizer (Mozobil). *Biochem Pharmacol* 2009; 77: 1655–1664.
8. Hendrix CW, Collier AC, Lederman MM *et al.* Safety, pharmacokinetics, and antiviral activity of AMD3100, a selective CXCR4 receptor inhibitor, in HIV-1 infection. *J Acquir Immune Defic Syndr* 2004; 37: 1253–1262.
9. DHHS Panel on Antiretroviral Guidelines for Adults and Adolescents. Guidelines for the Use of AntiretroviralAgents in HIV-1-Infected Adults and Adolescents. Available at: http://aidsinfo.nih.gov/contentfiles/AdultandAdolescentGL.pdf (last accessed 30 May 2012).
10. European AIDS Clinical Society. Clinical management and treatment of HIV-infected adults in Europe. Version 6, October 2011. Available at: http://www.europeanaidsclinicalsociety.org/images/stories/EACS-Pdf/eacsguidelines-6.pdf
11. Vandekerckhove L, Wensing A, Kaiser R *et al.* European guidelines on the clinical management of HIV-1 tropism testing. *Lancet Infect Dis* 2011; 11: 394–407.
12. Coakley E, Reeves JD, Huang W *et al.* Comparison of human immunodeficiency virus type 1 tropism profiles in clinical samples by the Trofile and MT-2 assays. *Antimicrob Agents Chemother* 2009; 53: 4686–4693.
13. Sing T, Low AJ, Beerenwinkel N *et al.* Predicting HIV coreceptor usage on the basis of genetic and clinical covariates. *Antivir Ther* 2007; 12: 1097–1106.
14. Poveda E, Seclen E, Gonzalez MM *et al.* Design and validation of new genotypic tools for easy and reliable estimation of HIV tropism before using CCR5 antagonists. *J Antimicrob Chemother* 2009; 63: 1006–1010.
15. Prosperi MC, Bracciale L, Fabbiani M *et al.* Comparative determination of HIV-1 co-receptor tropism by Enhanced Sensitivity Trofile, gp120 V3-loop RNA and DNA genotyping. *Retrovirol* 2010; 7: 56.
16. Simon B, Grabmeier-Pfistershammer K, Rieger A *et al.* HIV coreceptor tropism in antiretroviral treatment-naive patients newly diagnosed at a late stage of HIV infection. *AIDS* 2010; 24: 2051–2058.
17. Svicher V, D'Arrigo R, Alteri C *et al.* Performance of genotypic tropism testing in clinical practice using the enhanced sensitivity version of Trofile as reference assay: results from the OSCAR Study Group. *New Microbiol* 2010; 33: 195–206.
18. Wilgenbusch JC, Swofford D. Inferring evolutionary trees with PAUP*. In: *Curr Protoc Bioinformatics*, Chapter 6, Unit 6.4. 2003.
19. Posada D, Buckley TR. Model selection and model averaging in phylogenetics: advantages of akaike information criterion and bayesian approaches over likelihood ratio tests. *Syst Biol* 2004; 53: 793–808.
20. Hochberg Y, Benjamini Y. More powerful procedures for multiple significance testing. *Stat Med* 1990; 9: 811–818.
21. Svicher V, Balestra E, Cento V *et al.* HIV-1 dual/mixed tropic isolates show different genetic and phenotypic characteristics and response to maraviroc in vitro. *Antiviral Res* 2011; 90: 42–53.
22. Svicher V, Altreri C, Artese A *et al.* Identification and structural characterization of novel genetic elements in HIV-1 -V3 loop regulating co-receptor usage. *Antivir Ther* 2011; 16: 1035–1045.
23. Shimizu N, Haraguchi Y, Takeuchi Y *et al.* Changes in and discrepancies between cell tropisms and coreceptor uses of human immunodeficiency virus type 1 induced by single point mutations at the V3 tip of the env protein. *Virology* 1999; 259: 224–333.
24. Briggs D, Tuttle D, Sleasman J, Goodenow M. Envelope V3 amino acid sequence predicts HIV-1 phenotype (co-receptor usage and tropism for macrophages). *AIDS* 2000; 14: 2937–2939.
25. Hu Q, Trent J, Tomaras G *et al.* Identification of ENV determinants in V3 that influence the molecular anatomy of CCR5 utilization. *J Mol Biol* 2000; 302: 359–375.

26. Carrillo A, Ratner L. Human immunodeficiency virus type 1 tropism for T-lymphoid cell lines: role of the V3 loop and C4 envelope determinants. *J Virol* 1996; 70: 1301–1309.
27. Ross TM, Cullen BR. The ability of HIV type 1 to use CCR-3 as a co-receptor is controlled by envelope V1/V2 sequences acting in conjunction with a CCR-5 tropic V3 loop. *Proc Natl Acad Sci* 1998; 95: 7682–7686.
28. Dimonte S, Mercurio F, Svicher V, D'Arrigo R, Perno CF, Ceccherini-Silberstein F. Selected amino acid mutations in HIV-1 B subtype gp41 are associated with specific gp120v_3 signatures in the regulation of co-receptor usage. *Retrovirol* 2011; 12: 8.
29. Poveda E, Briz V, de Mendoza C *et al.* Prevalence of X4 tropic HIV-1 variants in patients with differences in disease stage and exposure to antiretroviral therapy. *J Med Virol* 2007; 79: 1040–1046.