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Performance of genotypic tropism testing in clinical practice using the enhanced sensitivity version of Trofile as reference assay: results from the OSCAR Study Group

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SUMMARY

Objective: The goal of the OSCAR programme is to evaluate the performances of genotypic HIV-1 tropism testing in clinical practice using the enhanced sensitivity version of Trofile (ESTA) as reference-assay.
Methods: HIV-1 coreceptor-usage was assessed using plasma samples from 406 HIV-1 infected patients by ESTA and by gp120 V3 population-sequencing followed by Geno2pheno (set at a False Positive Rate [FPR] of 10% and 5%).
Results: ESTA was successful in 365 (89.9%) samples indicating R5 in 254 (69.6%), and DM/X4 in 111 (30.4% of samples (104 [28.5%] DM and 7 [1.9%] X4). Genotypic-testing successfully assessed viral tropism for all 406 samples, including the 41 with undetermined result by ESTA. Genotypic-tropism testing at a FPR of 5% and 10% was 81.1% and 78.4% concordant with ESTA, respectively. Despite a sensitivity of 48.7% and 55.9% at a FPR of 5% and 10%, respectively, a high concordance (specificity: 95.3% for FPR of 5% and 88.2% for FPR of 10%) between genotypic-tropism testing and ESTA was reached in the detection of R5-tropic viruses.
Conclusion: Our results are in line with other European studies, and support the routine use of genotypic tropism testing in clinical-settings for monitoring of HIV-1 infected patients candidate to or failing CCR5-antagonists.

KEY WORDS: HIV, Tropism, V3 loop, Genotypic tropism testing, Trofile

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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 a family chemokine receptors, mainly CCR5 or CXCR4. The V3 loop in HIV-1 gp120 has been shown to be critical for co-receptor binding (Jensen *et al.,* 2003). HIV-1 strains can be phenotypically classified according to virus ability to use the CCR5 (R5) and/or CXCR4 (X4) co-receptor. Thus, pure R5-tropic and pure X4-tropic virus can use only the CCR5 and CXCR4 co-receptors to enter the target cell, respectively (Berger *et al.,* 1998), while dual-tropic virus can use both co-receptors. In a virus population, the use of both co-receptors can be due either to the presence of dual-tropic clones or to a mixture of pure R5-tropic and X4-tropic clones or both. This is cumulatively defined as dual/mixed phenotype.

HIV-1 co-receptor usage is of central pathological and clinical importance. Indeed, it has been shown that R5-tropic viruses are generally responsible for the establishment of the initial infection and predominate in the majority of newly HIV-1 infected patients, while the use of the CXCR4 co-receptor is generally seen in more advanced stages of disease, and has been associated with a more rapid CD4 decline and progression to AIDS (Regoes *et al.,* 2005; Berger *et al.,* 1998). In addition, CCR5-antagonists are a new class of anti-HIV-1 drugs that specifically inhibit the entry of CCR5-tropic HIV-1 strains into the target cells by allosteric inhibition of the CCR5 co-receptor, (Dorr *et al.,* 1995; Regoes *et al.,* 2005; Princen *et al.*, 2005). Maraviroc is the first approved CCR5 antagonist, that entered clinical practice in 2007. The determination of HIV-1 tropism is mandatory before the prescription of CCR5 antagonists. In particular, the recent guidelines recommend to use a co-receptor tropism assay whenever the use of a CCR5 antagonist is being considered (DHHS guidelines). However, the definition of methodologies for a correct determination of HIV-1 co-receptor usage in clinical practice is challenging. So far, several phenotypic assays have been developed to determine HIV-1 tropism in clinical samples. Among them, the original Trofile assay (Monogram Biosciences), with a 10% sensitivity threshold for detection of minority X4 virus population, has been used to screen patients for inclusion in clinical trials of CCR5 antagonists (Whitcomb *et al.,* 2007; Gulick *et al.,* 2007; Gulick *et al.,* 2008; Fätkenheuer *et al.,* 2008; Saag *et al.,* 2009; Soriano *et al.,* 2009). In 2008, an enhanced sensitivity version of Trofile (ESTA) has been set up with a lower limit of sensitivity for detecting minority X4 virus of 0.3% in viral clone mixtures (Reeves *et al.,* 2009). However, the sensitivity of ESTA with clinical samples at different levels of viremia is still poorly documented.

Phenotypic assays are complex, provided as a remote service by specialized companies and further made unpractical by high cost and long turnaround times. In addition, most of them cannot determine HIV-1 tropism in clinical samples with viremia below 1,000 copies/ml and thus cannot be used in the context of virological failure with low viremia.

For all these reasons, genotypic tropism assays are increasingly being used. They are based on the population-based sequencing of the patient-derived HIV-1 gp120 V3 domain, which is the major determinant for co-receptor binding. The genetic information contained in the V3 sequence is then used to infer HIV-1 tropism by using web-based bioinformatic interpretation tools. Genotypic tropism assays have the great potential for a routinely assessment of HIV-1 tropism, since they are more widely available, relatively inexpensive and more rapid to perform than phenotypic assays. In addition, they can be used either on plasma (through the amplification of viral RNA) or on peripheral blood mononuclear cells (PBMCs) (through the amplification of proviral DNA), thus representing a feasible option for tropism determination in the context of undetectable viremia, such as simplification and intensification therapeutic strategies.

Current data indicate that genotypic assays can

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be a valuable tool for assessing HIV-1 tropism to the point that they are already and routinely used in clinical practice in several European countries, such as United Kingdom, Germany, and France (http://www.viro.med.uni-erlangen.de/nrz/recommendation080324.pdf; http://www.bhiva.org/ClinicalGuidelines.aspx). In Italy, studies addressing this point at a national level are still missing.

In this context, the OSCAR (**O**ptimizing the **S**usceptibility to **C**CR5 **A**ntagonists **R**esponse) study was aimed at designing a new protocol for V3 sequencing and at defining the performances of genotypic tropism testing in clinical settings by using as ESTA as reference assay. To fulfill this goal, a pan-Italian network of clinicians and virologists has been established throughout Italy that has allowed the collection of one of the largest cohort of patients with matched genotypic and phenotypic Tropism results.

METHODS

Study population

This study includes 406 plasma samples from HIV-1 infected patients, with plasma HIV-1 RNA >100 copies/ml and naïve to maraviroc, enrolled at 20 clinical centers throughout Italy in the framework of the OSCAR programme. For each patient, HIV-1 tropism was assessed both phenotypically by ESTA and genotypically by V3 sequencing and tropism prediction. Plasma samples for genotypic and phenotypic determination of HIV-1 tropism were collected at the same timepoint. Demographic information, viral load, CD4 counts and antiretroviral treatment exposure were recorded at the time of the analysis.

Phenotypic characterization of HIV-1 co-receptor usage

ESTA was used for the phenotypic determination of HIV-1 co-receptor usage. Briefly, the patient's derived *env* gene was amplified from plasma HIV-1 RNA and cloned into an expression vector, that is then co-transfected together with an env-deficient HIV genomic vector carrying a luciferase reporter gene into human embryonic kidney cells (HEK293). The resulting pseudo-typed HIV particles were harvested and inoculated onto CXCR4-expressing and CCR5-expressing U87 cells. Samples that produced a signal (measured as relative light units in a luciferase assay) only on CCR5-expressing cells were considered pure R5-tropic, and those that produced a signal only on CXCR4-expressing cells were considered pure X4-tropic; those that produced a signal on both cell types are considered dual or mixed tropic (D/M). Signal ablation with specific co-receptor inhibitors is used to confirm the assigned tropism.

Sequencing of HIV-1 gp120 V3 domain

The OSCAR programme was specifically designed to set up a new protocol for population-based sequencing of the V3 loop. The protocol has been designed and optimized as follows. HIV-1 RNA was extracted on plasma samples by means of a commercially available kit (QIAamp RNA Viral Mini kit, Qiagen) according to the manufacturer's instructions. The V3-containing region of the *env* gene was then reverse-transcribed and amplified using the forward primer V3S2 5'CAGCACAGTACAATGTACACA 3' (nucleotide [nt]: 630-650 of HIV-1 HXB2 gp120 env gene) and the reverse primer V3AS5 5'CTTCTCCAATTGTCCCTCA 3' (nt: 1292-1310) (Figure 1, Table 1). The conditions for reverse transcription and amplification were: one cycle at 50°C for 30 min, one cycle 94°C for 2 min, 40 cycles (94°C 30s, 52°C 30s, 72°C 40s), and a final step at 72°C for 10 min, using the following reaction mix: 25 ul of RNA template, 8 ul of 5 mM Mg++, 3 ul of Dnase Rnase free water, 0.75 ul of each primer at a concentration of 10 uM, 1 ul of Rnase out (40 U/ul), 1.5 ul of RT/Taq, 1 ul of dNTPs at a concentration of 10 mM for a total of 40 ul.

When the RT-PCR product was not visible on agarose gel, a semi-nested PCR was used with the inner forward primer V3S6 5'CTGTTAAATGGCAGTCTAGC3' (nt: 682-702) and the reverse primer V3AS5 (Figure 1, Table 1). Amplification conditions were: one cycle at 93°C for 12 min, 40 cycles (95°C 30s, 52°C 30s, 72°C 40s) and a final step at 72°C for 10 min, using the following reaction mix: 5 ul of buffer taq 10X, 4 ul of Mg++ at a concentration of 25 mM, 32.5 ul of Dnase Rnase free water, 0.9 ul of each primer at a concentration of 10 uM, 1 ul of dNTPs at a concentration of 10 mM, 0.7 ul of Taq (5 u/ul) for a total of 45 ul.

The PCR product was purified by Microcon PCR purification kit (Millipore). Negative and positive

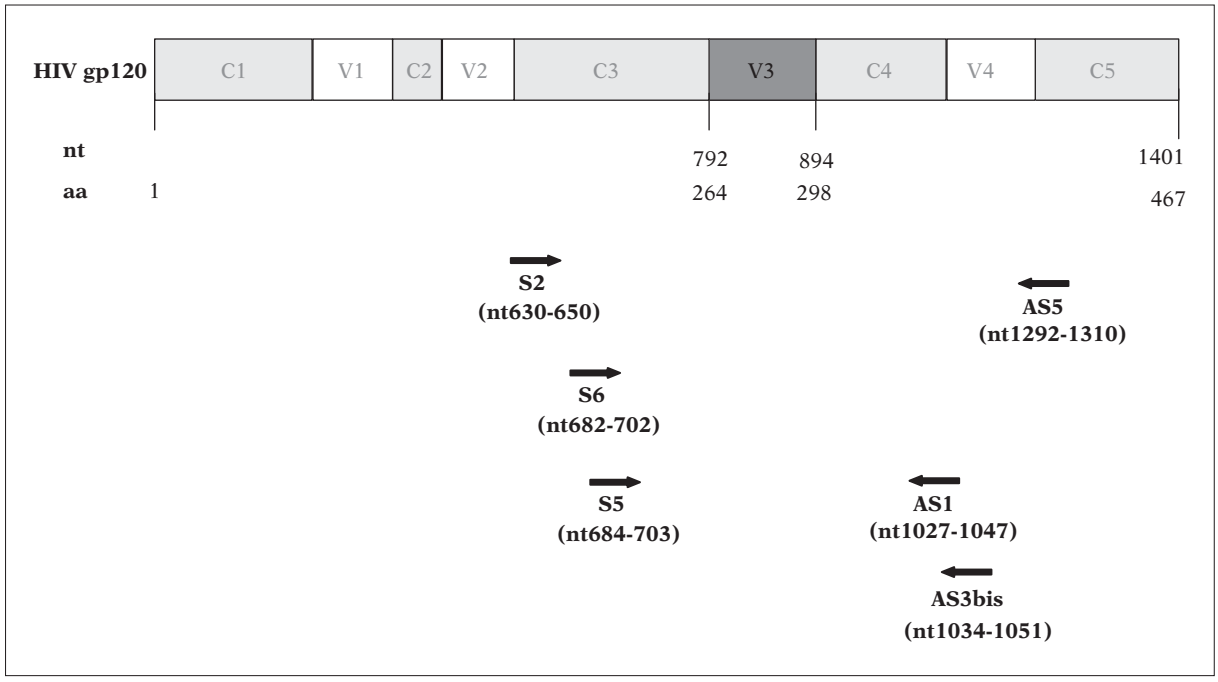

FIGURE 1 - *Location on HIV-1 gp120 coding region of the primers used for the amplification and sequencing of the V3 loop. Primers S2 and AS5 were used for the RT-PCR reaction. Heminested PCR was directed by primers S6 and AS5. Primers used for sequencing reaction are S6, S5, AS1, and AS3bis.*

control samples were included in each PCR run to exclude false-positive and false-negative reactions. PCR-products were then sequenced by using the BigDye terminator v.3.1 cycle sequencing kit (Applied-Biosystems), and an automated sequencer (ABI-3100). Four different overlapping sequence-specific primers were used to ensure the coverage of the V3-sequence by at least two sequence segments (Figure 1). The sequencing conditions were: one cycle 96°C 3 min, 25 cycles (96°C 30s, 50°C 10s, 60°C 4 min) and the following primers were used: V3S6 5'CTGTTAAATGGCAGTCTAGC 3', V3S5 5' GTTAAATGGCAGTCTAGCAG 3', V3AS1 5' GAAAAATTCCCCTCCACAATT 3' and V3AS3bis 5' CAATTTCTGGGTCCCCTC 3' (Figure 1, Table 1).

For a subset of samples, some modifications of the above-mentioned protocol were followed. In

TABLE 1 - *Overview of the primer and cycling conditions for V3 PCR.*

	PCR			*Nested PCR*		
Sense primer	5'-CAGCACAGTACAATGTACACA-3'			5'- CTGTTAAATGGCAGTCTAGC -3'		
Antisense primer	5'-CTTCTCCAATTGTCCCTCA- 3'			5'-CTTCTCCAATTGTCCCTCA-3'		
PCR settings	30 mins	50°C,	1 cycle	12 mins		
	2 mins	94°C,	1 cycle		93°C,	1 cycle
	30s	94°C		30s	95°C	
	30s	52°C,	40 cycles	30s	52°C	40 cycles
	40s	72°C		40s	72°C	
	10 min	72°C	1 cycle	10 min	72°C	1 cycle

PCR, polymerase chain reaction.

particular, thirthy five plasma samples was sequenced with an alternative method. Briefly RT-PCR was carried out with a 40 µl volume containing 10 µl sample RNA. 16 µl of MMix1, that contain 0.7 µl forward primer V3S2 (5' CAGCACAGTACAATGTACACA 3') and reverse primer V3AS5 (5' CTTCTCCAATTGTCCCTCA 3') [15 µM], 1.8 µl dNTPs [10 mM], 1.2 µl dithiothreitol (DTT), 0.6 µl RNase Inhibitor [40 U/µl], 14.5 µl steril water, was mixed with the sample. The Second MMix (11.7 µl RT-PCR buffer, 06 µl Rnase-inhibitor, 1.2 µl RT Enzyme and 2.9 µl Dna Polymerase) was added after 10 min at 50°C. RT-PCR was run with the following temperature program: 20 min at 50°C and 2 min at 94°C, followed by 40 cycles with 30 s at 94°C, 30 s at 57°C, and 120 s at 68°C and a final extension for 7 min at 68°C.
CLIP sequencing reaction was performed by using a 7-deaza-dGTP Cy5/Cy5.5 dye primer cycle sequencing kit (Siemens Healthcare Diagnostics, Milan Italy) according to the manufacturer's instructions. The four CLIP reaction mixtures contained 2.8 µl CLIP buffer, 8.8 µl molecular water, 2.8 µl forward primer V3S6 (5'-Cy5.5-CTGTTAAATGGCAGTCTAGC-3') and reverse primer V3AS3bis (5'-Cy5-5'CAATTTCTGCCCCTC GGT-3') [3 µM], 5 l sample cDNA, 3 µl of the four terminator nucleotides, and 4.4 µl Thermosequenasi Enzyme [32U/µl]. The CLIP-specific cycling program was 5 min at 94°C, followed by 30 cycles of 20 s at 94°C, 20 s at 55°C, and 60 s 70°C and final extension for 7 min at 70°C. Thereafter, Stop Loading Dye (6 µl) was added. Samples were heated to 94°C for 3 min and incubated at 4°C. Fragments were separated on a TruGene Tower (Siemens Healthcare Diagnostics, Milan Italy) with a 6% polyacrylamide gel. Sequence data were acquired and analyzed using the OpenGene DNA Sequencing System and read against a newly created HIV-1 V3 loop sequence-specific reference library.

Genotypic prediction of viral tropism

HIV-1 co-receptor usage was inferred from the V3 nucleotide sequence by using the geno2pheno algorithm available at the following website http://coreceptor.bioinf.mpi-inf.mpg.de/. The system is based on a support vector machine methodology that has been trained with a set of V3 nucleotide sequences with known phenotypic tropism. The tool can also analyze amino-acid mixtures deduced from degenerate base calls. The result of the interpretation is given as a quantitative value, the false positive rate (FPR), that defines the probability of classifying an R5 virus falsely as X4.
HIV-1 co-receptor usage was inferred by using both the clonal and the clinical version of geno2pheno set at FPR of 10% and 5%. For clinical inference of viral tropism, viremia at the time of V3 sequencing and nadir of CD4 cell count were used.

Reproducibility of the test and validation of clinical center participating in the OSCAR programme

The OSCAR programme was designed to set up a new protocol for V3 sequencing to be shared with the majority of clinical and virological centers in Italy. For this reason, a validation procedure for each virological center has been set up. In particular, for each center, 10 plasma samples were processed in duplicate, one by the center to be validated and the other by the reference center. In particular, 4 reference centers have been identified in Rome, Milan, Siena, and Padova; these centers have been previously validated using the same above-mentioned procedure. The validation process was considered successful if the V3 nucleotide sequences obtained from the same plasma sample showed a degree of similarity of >95% and no change in HIV-1 tropism determination was observed. The validation was successful for all the 20 centers involved in the OSCAR programme.

Statistical analysis

To assess the performances of genotypic tropism testing, the sensitivity and specificity for the detection of X4 variants and their 95% confidence intervals (95% CIs) were calculated considering ESTA as reference assay. Sensitivity values were calculated as the proportion of samples that were considered as harboring X4-tropic viruses by genotypic test within the whole group of DM/X4 viruses detected by ESTA. Specificity values were reported as the proportion of patients with R5 viruses by genotype within all the R5 viruses by phenotypic test. Data were analyzed using the statistical software package SPSS (SPSS Inc., Chicago, IL).

Phylogenetic analysis

For each sample, HIV-1 subtype was determined by using the geno2pheno algorithm and confirmed by phylogenetic analysis of V3-containing nucleotide sequences. In particular, all the sequences were aligned and compared with reference sequences for the Major HIV-1 subtypes, available at: http://hiv-web.lanl.gov/content/hiv-db/SUBTYPE_REF/align.html using CLUSTAL X (Thompson *et al.,* 1994). The sequences were then manually edited by the Bioedit program (Hall *et al.,* 1999). Phylogenetic trees were generated using GTR Model of substitution with both NJ and Maximum Likelihood (ML) tree building methods (Swafford KL, 1999). The best fitting nucleotide substitution model was tested with the Hierarchical Likelihood Ratio Test (HLRT) implemented in the Model Test V3.0 software (Posada *et al.,* 1998). The statistical robustness within each phylogenetic tree was confirmed with a bootstrap analysis using 1000 replicates for the Neighbor-Joining (NJ) tree. All calculations were performed with PAUP* 4.0b10 (http://paup.csit.fsu.edu/about.html) software. For 39 patients, available pol sequences were used to confirm HIV-1 subtype by phylogenetic analysis (Alteri *et al.,* 2008). Phylogenetic analysis was also used to identify potential cross-contaminations during the sequencing process.

TABLE 2 - *Patients' characteristics.*

Number of patients	406
Male sex, number (%)	229 (56.4)
HIV-1 subtype, number (%)	
B	335 (82.5)
F	22 (5.4)
CRF02_AG	19 (4.7)
C	10 (2.5)
Other	20 (4.9)
HIV-1 RNA load (log copies/ml)	
Median	4.3
Interquartile range	3.5-4.9
CD4 cell count (cells/ul)	
Median	310
Interquartile range	193-464
Nadir CD4 cell count (cells/ul)	
Median	186
Interquartile range	53-320
Therapy status, number (%)	
Treatment-experienced	255 (62.8)
Drug-naive	79 (19.5)
Not available	72 (17.7)
Trofile high sensitivity, number (%)	
R5	254 (62.6)
X4	7 (1.7)
dual/mixed	104 (25.6)
not available	41 (10.1)

RESULTS

Patient's characteristics

This study included 406 patients with HIV-1 RNA >100 copies/ml and naïve to maraviroc. Their clinical and viro-immunological characteristics are shown in Table 2. At the time of sample collection, the median viremia was 4.3 (IQR: 3.5-4.9) $\log_{10}$ HIV-1 RNA copies/ml, and the median CD4 count was 310 (IQR:193-464) cells/ul. The large majority of patients harbored HIV-1 B subtype (N = 335, 82.5%). Other non-B subtypes or recombinant forms observed at a frequency >2% were: F (N = 22, 5.4%), CRF02_AG (N = 19, 4.7%), C (N = 10, 2.5%).

Therapy status was known for 334 (82.3%) patients. Among them, 255 (62.8%) were treatment-experienced patients. The proportion of antiretroviral-treated patients was similar in B and non-B subtypes (61.3% versus 67.1%). No statistically significant differences in viremia and CD4 cell count were observed between drug-naïve and treatment-experienced patients infected with HIV-1 B subtype (4.6 [3.8-5.0] $\log_{10}$ copies/ml versus 4.2 [3.4-4.9] $\log_{10}$ copies/ml, and 325 [164-523] $\log_{10}$ copies/ml versus 307 [193-450] $\log_{10}$ copies/ml, P>0.100).

Phenotypic characterization of HIV-1 co-receptor usage

Among the 406 plasma samples collected for the study, ESTA was obtained for 365 (89.9%) samples indicating R5 in 254 (69.6%), and DM/X4 in 111 (30.4% of samples (104 [28.5%] DM and 7 [1.9%] X4) (Table 2). A significant lower prevalence of pure R5-tropic viruses and a higher prevalence of DM-tropic viruses was observed in patients infected with HIV-1 B subtype than in those infected with non-B subtypes or recombinant forms (R5: 67.0% versus 82.3%, P=0.022;

DM: 30.7% versus 17.7%, P=0.045). All the 7 pure X4-tropic viruses were found in patients infected with HIV-1 B subtype.
In the set of HIV-1 B subtype infected patients, we observed a higher prevalence of R5-tropic viruses and a lower prevalence of DM/X4-tropic viruses in drug-naïve than in treatment-experienced patients, even if not statistically significant (R5: 74.5% versus 62.6%, DM/X4: 25.5% versus 37.4%, P>0.100).

Evaluation of the performance of genotypic tropism testing

Efficiency of V3 sequencing

The protocol for V3 sequencing set up in the framework of the OSCAR programme showed an overall efficiency of 84.2%. This efficiency varied according to the level of viremia (Figure 2). In particular, for viremia ranging between 100-500 copies/ml, the rate of successful sequencing was 63%, it progressively increased to 81% and 88% for viremia ranging between 500-1,000 copies/ml and 1,000-10,000 copies/ml, respectively, and reached ~95% for viremia >10,000 copies/ml (Figure 2). The efficiency of V3 sequencing was not affected by HIV-1 subtype.

Accuracy of genotypic tropism testing in detecting X4-tropic using ESTA as reference

We defined the accuracy of genotypic tropism testing in detecting X4-tropic viruses separately in the set of 303 matched genotypic and phenotypic results from patients infected with HIV-1 B subtype and in the set of 62 matched genotypic and phenotypic results from patients infected with non-B subtypes or recombinant forms (Table 3). We used the geno2pheno algorithm (both the clonal and the clinical version) as interpretation tool since it is so far recognized as the most reliable and robust algorithm for tropism determination. The geno2pheno algorithm provides a quantitative value, the FPR, that is a measure of

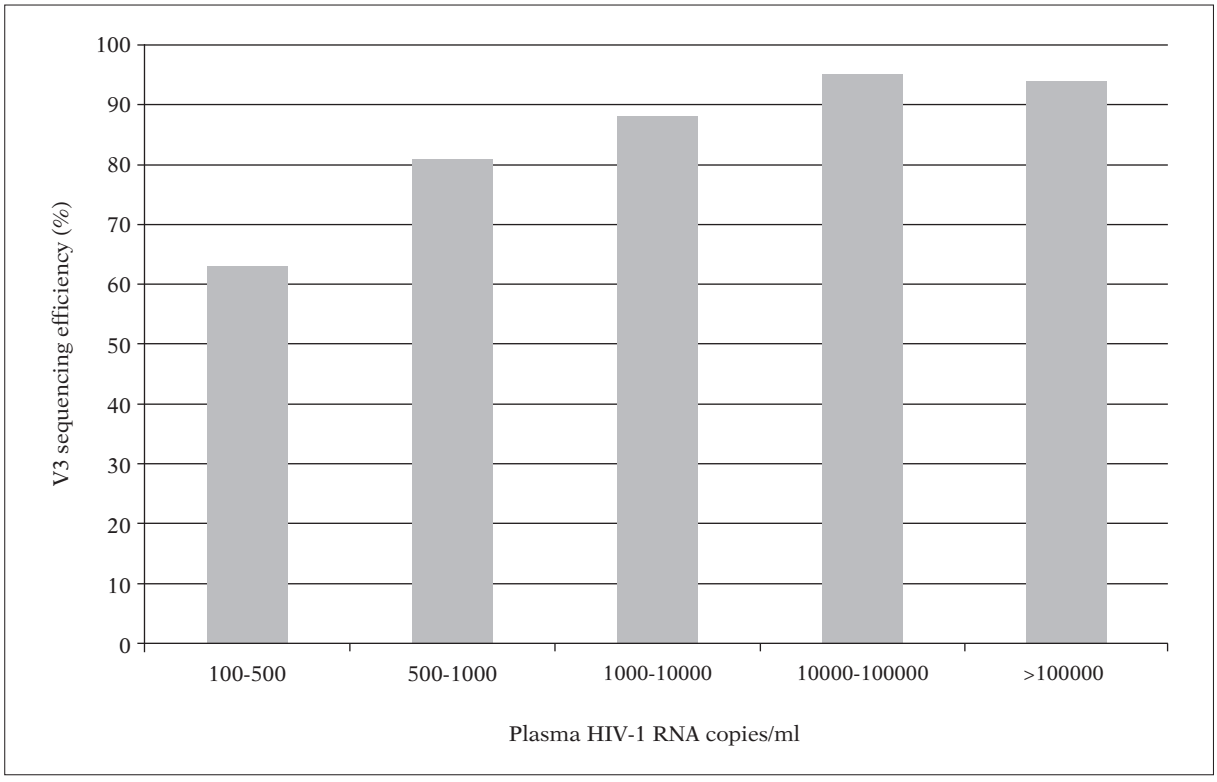

FIGURE 2 - *V3 sequencing efficiency stratified by viremia levels (HIV-1 RNA copies/ml). In order to provide a comprehensive determination of the efficiency of V3 sequencing, the rate of successful sequencing has been determined in a set of 592 samples, including samples involved in the OSCAR programme, and also other plasma samples tested so far for both clinical and research purposes.*

HIV-1 prediction to use the CCR5-coreceptor. We used as cut-off to discriminate between an R5-using or X4-using virus a FPR of 10%, proposed by the German Recommendations of the National Reference Centre for Retrovirus for determining HIV-1 co-receptor usage, and a FPR of 5%, recently used to analyze V3 sequencing obtained from patients enrolled in the Phase III Clinical trial Motivate (Harrigan *et al.,* 2009). If the FPR provided by geno2pheno for a specific V3 sequence is higher than the established cut-off, the prediction of HIV-1 co-receptor usage is R5. The accuracy measures of the clonal and clinical version of geno2pheno algorithm are now reported:
1) *Analysis of the clonal version of geno2pheno.* By analyzing the 303 matched genotypic and phenotypic results from patients infected with HIV-1 B subtype, we found that the overall concordance between ESTA and geno2pheno at a FPR of 5% and 10% was 80.5% and 77.6%, respectively (Table 3). The specificity (that measures the concordance between ESTA and genotypic tropism testing in detecting R5-viruses) was 96.0% and 89.0% and sensitivity (that measures the concordance in detecting DM/X4-viruses) was 49% and 55% when the FPR was set at 5% and 10%, respectively (Table 3). The performances of genotypic tropism testing in the set of 62 matched genotypic and phenotypic results from patients infected with HIV-1 non B subtypes were not significantly different from those observed for B subtype (Table 3).
2) *Analysis of the clinical version of geno2pheno.* In a subset of 148 HIV-1 B subtype infected patients,

TABLE 3 - *Performances of genotypic tropism testing using the enhanced sensitivity version of Trofile as reference assay*

Geno2pheno algorithm Clonal Version		*ESTA*				*Concordance %*	*Sensitivity (95%CI)*	*Specificity (95%CI)*	*Accuracy (95%CI)*
		R5	*D/M*	*X4*	*Total*				
B subtype									
	R5	195	50	1	246	80.5	0.49	0.96	0.72
FPR 5%	X4	8	43	6	57		(0.39-0.59)	(0.93-0.99)	(0.65-0.78)
	Total	203	93	7	303				
	R5	180	44	1	225	77.6	0.55	0.89	0.72
FPR 10%	X4	23	49	6	78		(0.45-0.65)	(0.84-0.93)	(66-79)
	Total	203	93	7	303				
Non-B subtypes									
	R5	47	6	0	53	83.9	0.45	0.92	0.69
FPR 5%	X4	4	5	0	9		(0.16-0.75)	(0.85-0.99)	(0.49-0.88)
	Total	51	11	0	62				
FPR 10%	R5	44	4	0	48	82.3	0.64	0.86	0.75
	X4	7	7	0	14		(0.35-0.92)	(0.76-0.96)	(57-93)
	Total	51	11	0	62				
Geno2pheno algorithm Clinical Version[a]		*ESTA*				*Concordance %*	*Sensitivity (95%CI)*	*Specificity (95%CI)*	*Accuracy (95%CI)*
		R5	*D/M*	*X4*	*Total*				
FPR 5%	R5	93	30	1	124	75.7	0.34	0.92	0.74
	X4	8	16	0	24		(0.20-0.48)	(0.87-0.07)	(0.67-0.81)
	Total	101	46	1	148				
FPR 10%	R5	88	26	1	0	73.0	0.42	0.87	0.73
	X4	13	20	0	23		(0.28-0.57)	(0.81-0.94)	(0.66-0.80)
	Total	101	46	1	148				

[a]The performances of the clinical version of the geno2pheno algorithm have been evaluated in a subset of 148 HIV-1 B subtype infected patients.
Abbreviation: ESTA, Enhanced Sensitivity Version of Trofile.

we also evaluated the concordance between the clinical version of geno2pheno algorithm and ESTA (Table 3). Compared to the clonal version, the clinical version of geno2pheno takes into account the value of viremia at the time of V3 genotyping and the value of nadir CD4 cell count. The clinical version was 75.7% and 73.0% concordant with ESTA when the FPR was set at 5% and 10%, respectively, with a specificity ranging from 92% to 87% and a sensitivity ranging from 34% to 42%, respectively. Thus, in our dataset, the performances of the clinical version were not significantly higher than that observed for the clonal one.

DISCUSSION

The OSCAR study has evaluated the performances of a genotypic tropism testing in clinical settings using ESTA as reference assay in one of the largest set of matched genotypic and phenotypic results collected so far. This has been feasible by the establishment of an extensive network of clinicians and virologists throughout Italy that has allowed the collection and analysis of 406 matched genotypic and phenotypic results. In line with other European studies, this study supports the reliability and the use of genotypic tool for tropism determination in clinical practice.

Several studies have evaluated the performances of genotypic tropism testing using the original version of Trofile or other phenotypic assays as reference (Low *et al.,* 2007; Poveda *et al.,* 2007; Raymond *et al.,* 2008; Poveda *et al.,* 2009; Chueca *et al.,* 2009; Harrigan *et al.,* 2009; Seclen *et al.,* 2009). The performances of genotypic tropism testing in comparison to ESTA have been assessed only in a very limited number of studies using a limited number of patients (Strang *et al.,* 2009, De Luca *et al.,* 2009, Sanchez *et al.,* 2009). Our and the above-mentioned studies are in agreement regarding the high concordance between genotypic and phenotypic tropism testing (including ESTA) in the detection of R5-tropic viruses. For instance, the specificity observed in our dataset ranges from 86% to 96% when the clonal version of geno2pheno has been used. Conversely, a lower concordance is generally observed in the detection of DM/X4 viruses (sensitivity in our cohort ranging from 42% to 64%). This result can be explained either by the fact that genotypic tropism testing cannot detect X4-tropic viruses representing less than ~10-20% of viral population, or by the fact that the current algorithms for HIV-1 tropism prediction do not take into account additional regions in the *env* gene, outside the V3 loop. Even if the clinical relevance of including additional regions remains to be established, previous studies have demonstrated that mutations in the V1/V2 loops and the C4 region of the gp120 are involved in mechanisms underlying co-receptor binding and usage (Suphaphiphat *et al.*, 2010; Pastore *et al.,* 2006). A recent study has also reported an increase in the accuracy of interpretation when both V2 and V3 were used compared to V2 or V3 alone (Thielen *et al.,* 2007).

Despite this lower sensitivity, recent studies have clearly shown that genotypic tropism testing is comparable with Trofile assay in predicting virological response to a maraviroc containing HAART regimen. In particular, the virological response to a maraviroc-containing regimen has been retrospectively predicted using the original version of Trofile and population-based V3 sequencing in a cohort of treatment-experienced patients enrolled in the framework of two phase III clinical trials MOTIVATE 1 and 2 and in the 1029 study (Harrigan *et al.,* 2009). Both Trofile and population-based V3 sequencing were equally successful in predicting virological response at week 12 and 24. Indeed, the percentage of virological success (viremia <50 copies/ml) at week 24 was 46.4% and 46.1% when the baseline presence of R5-tropic viruses was determined by Trofile or V3-sequencing, respectively (Harrigan *et al.,* 2009). Similar results have been recently observed in drug-naive patients enrolled in the MERIT trials using ESTA and V3-sequencing (Mc Govern *et al.,* 2010). Thus, these studies (although not randomized) support the use of genotyping in clinical settings to select candidates for maraviroc treatment. In addition, the 1029 study has shown that 45% of patients classified as R5 by the original Trofile and then reclassified as D/M by ESTA reached virological success (viremia <50 copies/ml) at week 48 of a maraviroc-containing regimen. In particular, by using the ultra-deep sequencing methodology, a rapid decline of viremia (3 log) has been observed at week 12 in patients where X4-tropic viruses were present in less than 10% of the entire viral population (Swenson *et*

al., 2009). Another recent study have highlighted the ability of a maraviroc-containing regimen to suppress HIV-1 replication in presence of DM-tropic viruses (Garcia *et al.,* 2009; Garcia *et al.,* 2010). Thus, overall results suggest that the use CCR5 antagonists could be enlarged to select patients with DM-tropic viruses and underline the need of studies aimed at defining a more precise threshold of X4-tropic viruses below which a CCR5 antagonist together with other active drugs can be safely used in different clinical contexts. In this light, the use of highly sensitive assay for the determination of HIV-1 tropism (such as ESTA) could limit the number of patients that can benefit at least partially from treatment with a CCR5 antagonist.

Genotypic tropism testing has been also shown to be a valid and accurate assay in predicting the risk of clinical progression in both drug-naïve and treatment-experienced patients (Fouchier *et al.,* 1992; De Jong *et al.,* 1992; Brumme *et al.,* 2004; Delobel *et al.,* 1999; Lin *et al.,* 2010). In particular, in a prospective cohort study of patients initiating antiretroviral regimen, patients with X4-tropic viruses predicted by genotyping had earlier mortality after starting therapy despite achieving viral suppression to less than 500 copies/ml compared with patients with R5-viruses. Again, these results support the use of genotypic tropism testing can be a valuable tool in clinical practice not only to determine patients that can benefit CCR5 antagonist treatment but also for an optimized monitoring of HIV-1 infected patients.

The methodology of V3 sequencing that has been designed in the framework of the OSCAR programme is characterized by a high rate of efficiency even in presence of viremia lower than 1,000 copies/ml. Indeed, the rate of successful sequencing was 63% and 81% for viremia ranging from 100-500 copies/ml and 500-1,000 copies/ml, respectively, and reached ~95% for viremia >10,000 copies/ml. The availability of methodologies that can assess HIV-1 co-receptor usage even when viremia is low, is crucial since they can be used even in those antiretroviral-treated patients with persistently low, but detectable, levels of HIV-1 RNA in the range of 50-1000 copies/ml.

In addition, the methodology of V3 sequencing used in this study is based on a single round of amplification of the V3 region. In other studies, a triplicate round of amplification has been performed (Harrigan *et al.,* 2009; Mc Govern *et al.,* 2010). The use of single versus triplicate genotypic analysis is still matter of debate. Indeed, preliminary data from clinical trials have suggested that genotypic analysis in triplicate may increase the detection of X4-tropic viruses. However, a recent study has evaluated the effect of triplicate testing on genotypic tropism prediction in routine clinical practice and has found that the triplicate testing resulted in an enhanced detection of X4-variants only in a small percentage of patients (Wensing *et al.,* 2010). Further studies are necessary to evaluate the clinical significance of triplicate versus single genotypic analysis for tropism prediction.

In conclusion, in line with other European studies, our study supports the routine use of genotypic tropism testing in clinical settings for monitoring HIV-1 infected patients and for a better tuning of in vivo efficacy of CCR5 antagonists.

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