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Performance of genotypic tropism testing on proviral DNA in clinical practice: results from the DIVA Study Group

Valentina Svicher[1], Claudia Alteri[1], Marco Montano[1], Roberta D'Arrigo[2], Massimo Andreoni[1], Gioacchino Angarano[3], Andrea Antinori[2], Guido Antonelli[4], Tiziano Allice[5], Patrizia Bagnarelli[6], Fausto Baldanti[7], Ada Bertoli[1], Marco Borderi[8], Enzo Boeri[9], Isabella Bon[8], Bianca Bruzzone[10], Anna Paola Callegaro[11], Maria Rosaria Capobianchi[2], Giampiero Carosi[12], Roberto Cauda[13], Francesca Ceccherini-Silberstein[1], Massimo Clementi[9], Antonio Chirianni[14], Manuela Colafigli[13], Antonella D'Arminio Monforte[15], Andrea De Luca[16], Antonio Di Biagio[10], Giuseppe Di Nicuolo[14], Giovanni Di Perri[5], Massimo Di Pietro[17], Fabiola Di Santo[1], Lavinia Fabeni[2], Giovanni Fadda[18], Massimo Galli[19], William Gennari[20], Valeria Ghisetti[5], Andrea Giacometti[6], Caterina Gori[2], Andrea Gori[21], Roberto Gulminetti[7], Francesco Leoncini[22], Gaetano Maffongelli[1], Franco Maggiolo[11], Giuseppe Manca[12], Franco Gargiulo[12], Canio Martinelli[22], Renato Maserati[7], Francesco Mazzotta[17], Genny Meini[23], Valeria Micheli[19], Laura Monno[24], Cristina Mussini[20], Pasquale Narciso[2], Silvia Nozza[25], Stefania Paolucci[7], Giorgio Palù[26], Saverio Parisi[26], Giustino Parruti[27], Angela Rosa Pignataro[9], Michela Pollicita[1], Tiziana Quirino[28], Maria Carla Re[8], Giuliano Rizzardini[19], Rosaria Santangelo[18], Renzo Scaggiante[26], Gaetana Sterrantino[22], Ombretta Turriziani[4], Maria Linda Vatteroni[29], Laura Vecchi[21], Claudio Viscoli[10], Vincenzo Vullo[4], Maurizio Zazzi[23], Adriano Lazzarin[25], Carlo Federico Perno[1]* on behalf of DIVA group

[1]University of Rome "Tor Vergata", Department of Experimental Medicine, Roma, Italy; [2]INMI "L. Spallanzani", Sequencing and Antiviral Drug Monitoring Unit, Roma, Italy; [3]Bari University, Clinic of Infectious Diseases, Bari, Italy; [4]"Sapienza" University of Rome, Italy; [5]University of Turin, Microbiology and Virology Laboratory, Torino, Italy; [6]Marche Politechnic University Medical School, Department of Biochemical Sciences and Public Health, Ancona, Italy; [7]Molecular Virology Unit, Virology and Microbiology, Fondazione IRCCS Policlinico San Matteo, Pavia; [8]University of Bologna, Section of Microbiology of the Department of Hematology and Oncologic Science, Bologna, Italy; [9]Vita-Salute San Raffaele University Laboratory of Microbiology and Virology, Milan, Italy; [10]San Martino Hospital, Genova, Italy; [11]Ospedali Riuniti, Department of Infectious Diseases, Bergamo, Italy; [12]Ospedali Civili di Brescia, Italy; [13]Catholic University of Sacred Heart, Institute of Clinical Infectious Diseases, Rome, Italy; [14]III Division of Infectious Diseases A.O.R.N. Cotugno, Naples, Italy; [15]"S. Paolo" Hospital, Milano, Italy; [16]U.O.C. Malattie Infettive Universitarie, Azienda Ospedaliera Universitaria Senese, Siena; [17]"S.M. Annunziata" Hospital, Firenze, Italy; [18]Catholic University of Sacred Heart, Institute of Microbiology, Rome, Italy; [19]"L. Sacco Hospital", Institute of Infectious and Tropical Diseases, Milan, Italy; [20]Modena University Hospital, Unit of Microbiology, Modena, Italy; [21]Division of Infectious Diseases, Department of Internal Medicine, San Gerardo Hospital, University of Milano-Bicocca, Monza, Italy; [22]"Careggi" Hospital, Firenze, Italy; [23]Department of Biotechnology, University of Siena, Italy; [24]University of Bari, Clinic of Infectious Diseases, Bari, Italy; [25]"S. Raffaele" Scientific Institute, Department of Infectious Diseases, Milano, Italy; [26]Padova University, Department of Histology, Microbiology and Medical Biotechnology, Padova, Italy; [27]Infectious Disease Unit, Pescara General Hospital, Pescara, Italy; [28]Busto Arsizio Hospital, Busto Arsizio (MI), Italy; [29]Pisa University, Virology Unit A.O.U. Pisana, Italy

SUMMARY

Objective: The DIVA study is aimed at setting up a standardized genotypic tropism-testing on proviral-DNA for the routine clinical diagnostic-laboratory. *Methods:* Twelve local centres and 5 reference centres (previously cross-validated) were identified. For inter-center validation-procedure, 60 peripheral-blood mononuclear cells (PBMCs) aliquots from 45 HAART-treated patients were randomly chosen for population V3 sequencing on proviral-DNA at local HIV centre and at reference-laboratory. Viral tropism was predicted by Geno2Pheno algorithm (False Positive Rate [FPR] = 20%) as proposed by the European-Guidelines. Quantification of total HIV-1 DNA was based on a method described by Viard (2004). *Results:* Quantification of HIV-1 DNA was available for 35/45 (77.8%) samples, and gave a median value of 598 (IQR:252-1,203) copies/10^6 PBMCs. A total of 56/60 (93.3%) samples were successfully amplified by both the reference and the local virological centers. The overall concordance of tropism prediction between local and reference centers was 54/56 (96.4%). Results of tropism prediction by local centers were: 33/54 (61.1%) R5 and 21/54 (38.9%) X4/DM. *Conclusion:* There was high concordance in the genotypic tropism prediction based on proviral DNA among different virological centers throughout Italy. Our results are in line with other European studies, and support the use of genotypic tropism testing on proviral DNA in patients with suppressed plasma HIV-1 RNA candidate to CCR5-antagonist treatment.

KEY WORDS: HIV, Proviral DNA, Tropism

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INTRODUCTION

Human immunodeficiency virus type 1 (HIV-1) entry into host cells requires coordinated interactions of the envelope glycoprotein gp120 with the CD4 receptor and with one of the chemokine receptors, CCR5 or CXCR4. Pure CCR5-tropic and pure CXCR4-tropic virus can use only the CCR5 and CXCR4 co-receptors to enter target cells, respectively, while dual-tropic virus can use both co-receptors (Berger *et al.*, 1998).
The study of HIV-1 co-receptor usage has pathogenetic implications due to its strong correlation with the rate of disease progression in HIV-1 infected individuals (Roeges *et al.*, 2005; Watres *et al.*, 2008; Raymond *et al.*, 2010). Determining HIV-1 co-receptor usage is also critical since the CCR5 co-receptor has become the target of a new class of anti-HIV-1 drugs that specifically inhibit the entry of CCR5-tropic HIV-1 strains into the target cells (MacArthur *et al.*, 2008). Maraviroc is the first approved CCR5 antagonist, that entered clinical practice in 2007. Assessment of HIV-1 co-receptor usage is mandatory for the clinical use of this drug. Among the different approaches for tropism determination, genotypic population sequencing is the preferred method in Europe (Vandekerckhove *et al.*, 2011 European Guidelines for tropism).
The use of maraviroc is recommended for the treatment of antiretroviral-experienced patients failing prior regimens. However, the favorable pharmacokinetic properties and the good safety profile of this drug can further support its consideration as part of switch or simplification strategies in drug-treated patients with undetectable plasma HIV-1 RNA. In addition, as shown in maraviroc clinical trials, a potential immune benefit of maraviroc might encourage its use as part of intensification strategies in HIV-1-infected patients with impaired CD4 gains despite prolonged suppression of HIV replication with antiretroviral therapy (Cooper *et al.*, 2010).
The determination of HIV-1 co-receptor usage in patients with undetectable/low level viremia is challenging. Two approaches have so far been explored. The first relies on testing older stored plasma specimens collected before initiation of ART. An alternative approach might be testing current proviral DNA collected from peripheral blood mononuclear cells (PBMCs). Recent data suggest that using proviral HIV-1 DNA could be an option in these patients (Seclen *et al.*, 2001; Souliè *et al.*, 2011).
In this light, the DIVA (**D**NA Tropism **I**talian **V**alidation Concerted **A**ction) study was aimed at developing a well-standardized genotypic test for tropism determination in proviral DNA shared by the Italian scientific community. The availability of a rapid and cost-effective assay to determine tropism on proviral DNA is crucial to optimize the use of CCR5 antagonists in clinical practice.

Corresponding author
Carlo Federico Perno
Department of Experimental Medicine
University of Rome "Tor Vergata"
Via Montpellier, 1 - 00133 Rome Italy
E-mail: cf.perno@uniroma2.it

METHODS

Structure of the DIVA study: reproducibility of the test and validation of virological centers participating to the study

The DIVA study was designed to set up a new protocol for V3 sequencing on proviral DNA for the routine clinical diagnostic laboratory. The study included two consecutive steps. Phase 1 was aimed at cross-validating the genotypic tropism test on proviral DNA among the 5 reference centers involved in the project (University of Rome "Tor Vergata" , University of Rome "La Sapienza", "San Raffaele" Institute, University of Siena, University of Padova). For this phase, PBMCs from 8 HAART-treated patients with a HIV-1 RNA <50 copies/ml from at least 6 months were collected. For each patient, PBMCs were divided into 6 aliquots: 5 containing ~$4x10^6$ cells for V3 sequencing and tropism prediction, and 1 containing 10^6 cells for quantification of total HIV-1 DNA. The 5 aliquots per patient were processed for V3 sequencing by the 5 reference centers and the results were then compared.
Phase 2 was aimed at validating the methodology for tropism determination on proviral DNA among the 12 local virological centers involved in the project. For this phase, each reference center sent to a defined set of local virological centers PBMCs from 5 distinct patients. For each patient, PBMCs were divided into 3 aliquots: 2 containing

~4x10^6 cells for V3 sequencing and tropism prediction, and 1 containing 10^6 cells for quantification of total HIV-1 DNA. The 2 PBMCs aliquots were processed for V3 sequencing each by the local and the reference center, respectively, and results were then compared.
For both phase 1 and 2, the validation process was considered successful if the V3 amino acid sequences obtained from the same sample showed a degree of amino acid similarity >80% and no change in HIV-1 tropism determination was observed for 4 out of 5 samples tested.

Protocol for tropism determination on proviral DNA

Cells. Lympho-monocytic cells were obtained from the peripheral blood of HIV-1 infected patients by separation over a Ficoll-Hypaque gradient as previously described (Aquaro and Perno, 2005).

Quantification of total HIV-1 DNA. To quantify total HIV-1 DNA in PBMCs, the Real Time TaqMan protocol published by Viard *et al.*, 2004 was adapted to the Light Cycler (Roche Molecular Biochemicals, Indianapolis, IN). The cellular line 8E5, containing one copy of HIV-1 DNA integrated for each cell, was used to build a standard curve of seven dilutions (75,000-37,500-3,750-375-37.5-3.75-1.87 copies). The sensitivity of PCR is one copy of HIV-1 DNA per reaction (1 copy per reaction=13.3 copies/10^6 PBMCs). The HIV-1 DNA target was hybridized with TaqMan probe and read on channel F1/F2 of the Light Cycler. To verify DNA integrity, the reagents of LC control DNA kit (Roche Molecular Biochemicals) were used, amplifying a 110-bp fragment of human b-globin in the same reaction. This second internal control target was hybridized with FRET probe and read on channel F3/F2 of the Light Cycler. To verify the accuracy of the Real Time PCR result, different HIV-1 DNA standards (AIDS Research and Reference Reagent Program, DAIDS, NIAID, NIH: PCR Panel 001 from Dr. Shirley Kwok and Dr. Cindy Christopherson, Roche Molecular Systems) were also quantified (Sarmati *et al.*, 2007).

Sequencing of HIV-1 gp120 V3 domain. HIV-1 DNA was extracted from PBMCs by means of a commercially available kit (QIAamp DNA Viral Mini kit, Qiagen) according to the manufactur-

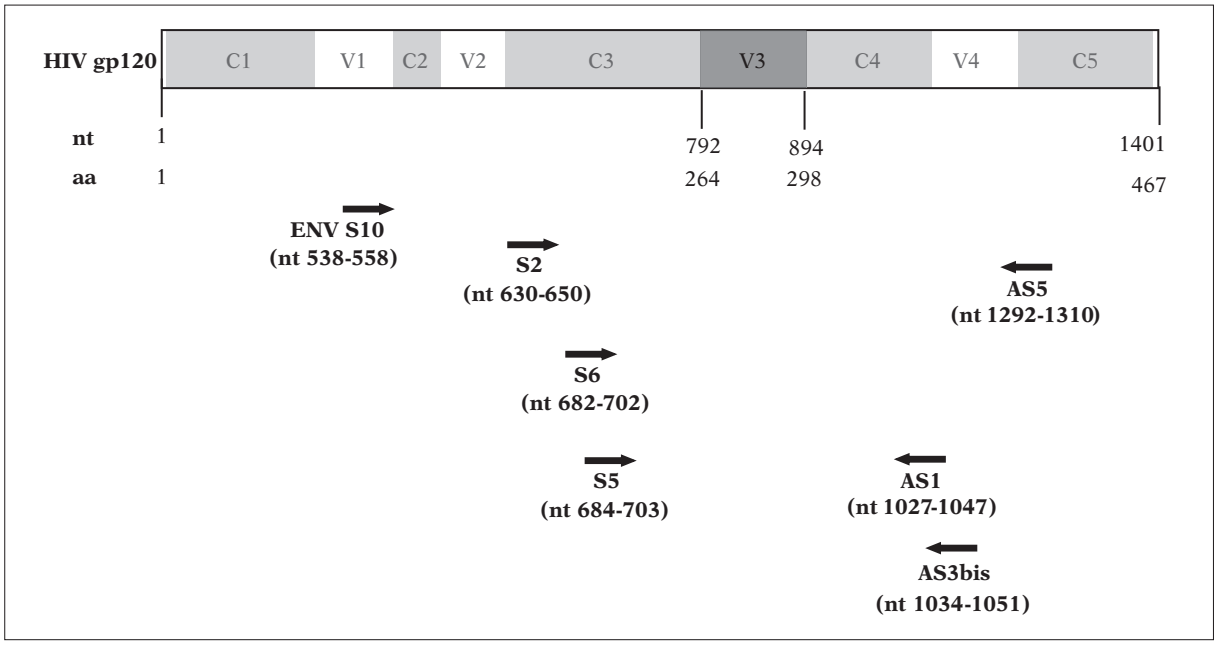

FIGURE 1 - *Location of the V3 loop amplification and sequencing primers on HIV-1 gp120 env coding region. Primers ENVS10 and AS5 were used for the first PCR reaction. Semi-nested PCR was directed by primers S2 and AS5. Primers used for sequencing reaction are S6, S5, AS1, and AS3bis.*

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

	PCR			*Nested PCR*		
Sense primer	5'- CCAATTCCCATACATTATTGT -3'			5'- CAGCACAGTACAATGTACACA -3'		
Antisense primer	5'-CTTCTCCAATTGTCCCTCA- 3'			5'-CTTCTCCAATTGTCCCTCA-3'		
PCR settings	12 min	93°C,	1 cycle	12 min	93°C,	1 cycle
	30s	93°C	}	30s	93°C	}
	30s	50°C	} 40 cycles	30s	51°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.

er's instructions. The V3-containing region of the env gene was amplified using the forward primer ENVS10 5'CCAATTCCCATACATTATTGT 3' (nucleotide [nt]: 538-558 of HIV-1 gp120 env gene) and the reverse primer V3AS5 5'CTTCTCCAATTGTCCCTCA 3' (nt: 1292-1310). The conditions for amplification were: one cycle 93°C for 12 min, 40 cycles (93°C 30s, 50°C 30s, 72°C 50s), and a final step at 72°C for 10 min, using the following master mix: 5 ul of Taq buffer 10X, 3 ul of 25 mM $MgCl_2$, 28.95 ul of DNase- and RNase-free bidistelled water, 0.75 ul of 10 uM primers, 0.8 ul of 12.5 mM dNTPs, 0.75 ul of Taq (5 u/ul) in a total volume of 40 ul.

When the first PCR product was not visible in agarose electrophoresis gels, a semi-nested PCR was run by using the following PCR primers (designed on HIV-1 gp120 consensus B sequence): the inner forward V3S2 5' CAGCACAGTACAATGTACACA 3' primer (nt: 630-650) and V3AS5 (Figure 1, Table 1). Amplification conditions were: one cycle at 93°C for 12 min, 40 cycles (93°C 30s, 51°C 30s, 72°C 50s) and a final step at 72°C for 10 min, using the following master mix: 5 ul of Taq Gold PE buffer 10X, 3 ul of 25 mM $MgCl_2$, 33.95 ul of DNase- and RNase-free bidistelled water, 0.75 ul of 10 uM primers, 0.8 ul of 12.5 mM dNTPs, 0.75 ul of Taq (5 u/ul) in a total volume of 45 ul.

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

For each sample, HIV-1 subtype was determined by using the geno2pheno algorithm and confirmed by phylogenetic analysis of V3-containing nucleotide sequences (Svicher *et al.*, 2010). Phylogenetic analysis was also used to identify potential cross-contaminations during the sequencing process.

Genotypic prediction of viral tropism. HIV-1 co-receptor usage was inferred from the V3 nucleotide sequence by using the geno2pheno algorithm available at 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 version of geno2pheno set at FPR of 20%.

RESULTS

Patient's characteristics. This study included a total of 53 HIV-1 infected patients (52 HAART-treated and 1 drug-naïve).
Among them, 49 (94.3%) had undetectable HIV-1 RNA (<50 copies/ml) and 2 had low plasma HIV-1 RNA (130 and 580 copies/ml).
For the remaining 2 patients, plasma HIV-1 RNA was 6,406 and 15,432 copies/ml, respectively.
Eight (all with plasma HIV-1 RNA <50 copies/ml) were included in the first phase and 45 in the second phase of the DIVA study.
Their clinical and viro-immunological characteristics are shown in Table 2.
At the time of sample collection, the median CD4 cell count was 495 (IQR:390-730) cells/ul, and the median total HIV-DNA (available for 42 out of 53 patients) was 638 (IQR:253-1,183) 10^6 PBMCs.
The large majority of patients harbored HIV-1 B subtype (N = 49, 92.4%).
Other non-B subtypes were: G (N = 3, 5.7%), and C (N = 1, 1.9%).

TABLE 2 - *Patients' characteristics*.

Number of patients	53
Male sex, number (%)	27 (65.8)
HIV-1 subtype, number (%)	
B	49 (92.4)
G	3 (5.7)
C	1 (1.9)
Total HIV-DNA (copies/10^6 cells)	
Median	638
Interquartile range	253-1183
CD4 cell count (cells/ul)	
Median	495
Interquartile range	390-730
Nadir CD4 cell count (cells/ul)	
Median	200
Interquartile range	105-348
Third drug, number (%)	
PI- treated	25 (62.5)
NNRTI-treated	15 (37.5)
N. of previous regimens	
Median	2
Interquartile range	1-3

Standardization of genotypic tropism testing on proviral DNA. The DIVA study was specifically designed to set up a standardized methodology for V3 sequencing on proviral DNA for the routine clinical diagnostic-laboratory. In order to achieve this goal, the study was articulated in 2 validation procedures whose results are reported as follows.

Results from the first phase. The first phase of the DIVA study was aimed at setting up and cross-validating the methodology of V3 sequencing on proviral DNA among the 5 reference centers enrolled in the project. For this phase, PBMCs from 8 HAART-treated patients with HIV-1 RNA <50 copies/ml from at least 6 months (median [IQR] time under virological suppression: 2 [3-4] years) were collected.
The median HIV-1 DNA was 391 (IQR: 256-807) copies/10^6 PBMCs (range 93-1,044 copies/10^6 PBMCs). There was complete agreement in tropism designation for all the 8 samples analyzed by the four reference centers (5 and 5 samples were scored as R5 and X4/DM, respectively). The median similarity among V3 sequences was 100% (IQR: 95%-100%). These results prompted the implementation of the second phase of the DIVA study.

Results from the second phase. The second phase was aimed at validating and sharing the methodology for tropism determination on proviral DNA among all the 12 local virological centers involved in the project. A total of 60 PBMCs aliquots from 45 HIV-infected patients were collected by the 5 reference centers and sent to local virological centers. All the samples sent to local centers were successfully amplified by the reference centers. Quantification of HIV-1 DNA (known for 35/45 patients) reported a median (IQR) HIV DNA: 598 (IQR:252-1,203) copies/10^6 PBMCs.
Fifty-six out of 60 (93.3%) PBMCs samples were successfully amplified by the local centers. The median HIV-1 DNA quantification for the not-amplifiable samples (available for 3/4 of them, all subtypes B) was 73, 212, 1,223 copies/10^6 PBMCs, respectively.
Tropism prediction obtained at the local and reference center was concordant for 54/56 (96.4%) samples. Genotypic tropism testing on proviral

DNA reported 33/54 (61.1%) R5 V3 sequences and 21/54 (38.9%) X4 V3 sequences.
The amino acid similarity and the difference in the FPR between V3 sequences obtained by the reference and local virological center were also estimated. The median (IQR) amino acid similarity was 95.7% (94.3%-98.6%), and the median difference (IQR) of FPR was 1.1 (-1.7;7.8). For 65.9% of samples, amino acid differences in V3 sequences obtained by local and reference center were due to the presence of at least 1 degenerate codon.
These results support the reliability of the methodology for V3 sequencing on proviral DNA set up in the framework of the DIVA study.

DISCUSSION

The DIVA study has allowed to set up a methodology for V3 sequencing on proviral DNA shared by Italian scientific community. The availability of a rapid and not expensive assay, as genotypic testing, to determine tropism in proviral DNA is crucial to optimize the use of CCR5 antagonists in clinical practice. Our results are in line with other European studies, and support the routine use of genotypic tropism testing on proviral-DNA in patients with suppressed plasma HIV-1 RNA candidate to CCR5-antagonist treatment.
Indeed, a recent study has evaluated the performances of genotypic tropism testing in proviral DNA using Toulouse Tropism Test phenotypic assay as reference in patients with primary HIV-1 infection (Raymond *et al.*, 2010). Genotypic tropism testing on proviral DNA using geno2pheno algorithm at a FPR of 10% and 5.75% was 96.3% and 100% concordant with phenotypic testing, respectively (Raymond *et al.*, 2010). Another recent study has shown a 85% concordance of genotypic tropism testing on proviral DNA with phenotypic tropism testing (at both 10 and 5% FPRs), supporting that genotypic tropism testing in proviral DNA offers a promising approach for tropism prediction in clinical practice (Verhofstede C *et al.*, 2011). Thus, as supported by the European Guidelines for the clinical management of tropism determination, the possibility to perform tropism testing on proviral DNA even during suppressed viremia can facilitate the use of CCR5 inhibitors as part of switching, simplification, or intensification strategies. In this light, the DIVA study has answered to a precise unmet medical need.
Several studies have investigated HIV-1 tropism in proviral DNA in HIV-1 infected viremic patients (Frange *et al.*, 2009; Verhofstede *et al.*, 2009; Seclen *et al.*, 2010; Swenson *et al.*, 2010). In general, these studies, mostly run in patients with detectable viremia, show a higher prevalence of X4-predicted sequences in proviral DNA than in RNA samples. It is unknown whether this is an overestimation of X4 variants in proviral DNA compared to plasma, due to the presence of less- or non-viable viral genomes that accumulated over the years without being represented in plasma (that is by definition enriched only with replicating, viable viral genomes). This situation may be typical of X4 viruses that, despite being randomly generated while virus replicates, are less represented in plasma than R5 viruses during the major part of the course of the disease. Specific studies are thus needed in order to better define the pathogenetic and clinical relevance of X4 species detected in proviral DNA, and to establish X4 frequency thresholds that can be tolerated in respect of effective CCR5 antagonist treatment.
In this study, we reported 33/54 (61.1%) R5 and 21/54 (38.9%) X4 V3 sequences using a threshold of FPR=20%, and 38/54 (70.4%) R5 and 16/34 (29.6%) X4 sequences using a threshold of FPR=10%. All these sequences were obtained from patients (all but 4) with undetectable viremia. The proportion of R5-using viruses in proviral DNA is consistent with two recent studies where a threshold of FPR=10% has been used. In particular, by analyzing a subset of 78 and 140 HAART-treated patients with suppressed viremia, R5 using viruses in proviral DNA were detected in 66.0% (Seclen *et al.*, 2010) and 70.0% (Soulie *et al.*, 2010) of patients analyzed, corroborating the critical role played by R5-tropic viruses in HIV pathogenesis. These 2 studies also showed a very low rate of switches from R5 to X4 tropism despite a median follow up >3 years of suppressive HAART, thus supporting the reliability of the genotypic tropism testing in proviral DNA.
The study by Soulie *et al.*, 2010 also highlighted a correlation between X4 tropism and nadir CD4 cell count. Indeed, X4-using viruses (although detected in all the nadir CD4 cell count categories

analyzed) were found with the highest prevalence in patients with nadir CD4 cell count <100 cells/ul. Consistent with these results, another study showed that the nadir CD4 cell count was the only baseline characteristic correlated with a switch from R5 to X4 tropism in proviral DNA (Saracino *et al.*, 2009). In our study, patients with X4-using strains are characterized by a nadir CD4 cell count lower than patients with CCR5-using strains, even if not statistically significant (148 [89-299] vs 243 [140-374], p=0.23).

Tropism prediction between local virological center and reference center was concordant for 54/56 (96.4%) samples. The 2 discordant samples had a total HIV-1 DNA <13 copies/10^6 PBMCs and 3,497 copies/10^6 PBMCs, respectively. For the former, the discordance in tropism prediction can be explained by stochastic errors in sampling and amplification in the setting of low viral input. For the latter, the discordance can be explained by the high degree of *env* genetic variability and quasispecies heterogeneity observed in proviral DNA acting as an archive of all the variants present in an infected patient during the natural history of the infection (Abbate *et al.*, 2010; Rozera *et al.*, 2009).

The methodology of V3 sequencing used in this study is based on a single amplification of the V3 region. 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 (Swenson *et al.*, 2010).

A recent study has evaluated the added value of triplicate versus single testing on genotypic tropism prediction in routine clinical practice in both plasma and proviral DNA samples. The authors found that the triplicate testing resulted in an enhanced detection of X4-variants only in a small percentage of patients, thus supporting the urgent need to compare single versus triplicate amplification procedures in relation to clinical outcome data (Symons *et al.*, 2011). Further studies are necessary to evaluate the clinical significance of triplicate versus single genotypic analysis for tropism prediction.

In conclusion, we showed high concordance in the genotypic approach for tropism-prediction on proviral-DNA among different virological centers throughout Italy. Our results support the feasibility and consistency of genotypic tropism testing on proviral DNA as a laboratory tool potentially assisting selection of patients with suppressed plasma HIV-1 RNA candidate to CCR5-antagonist treatment.

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Sterrantino, Canio Martinelli; *AOUPisana:* Maria Linda Vatteroni*; University of Siena (Siena)*: Maurizio Zazzi, Andrea De Luca, Genni Meini*; University of "Tor Vergata" (Rome)*: Carlo Federico Perno, Valentina Svicher, Claudia Alteri, Fabioloa Di Santo, Michela Pollicita, Massimo Andreoni Gaetano Maffongelli; Marco Montano; *I.N.M.I. "L. Spallanzani" (Rome)*: Andrea Antinori, Carlo Federico Perno, Roberta D'Arrigo, Maria Rosaria Capobianchi; *University of Rome "La Sapienza" (Rome)*: Vincenzo Vullo, Guido Antonelli, Ombretta Turriziani; *Catholic University "Sacro Cuore" (Rome)*: Roberto Cauda, Emanuela Colafigli, Giovanni Fadda, Rosaria Santangelo; *Pescara General Hospital*: Giustino Parruti; *Ospedale Cotugno*: Antonio Chirianni, Giuseppe Di Nicuolo; *University of Foggia and Bari*: Gioacchino Angarano, Laura Monno, Annalisa Saracino, Grazia Punzi.

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