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ORIGINAL RESEARCH

Concordance between HIV-1 genotypic coreceptor tropism predictions based on plasma RNA and proviral DNA

C Verhofstede,[1] D Brudney,[2] J Reynaerts,[1] D Vaira,[3] K Fransen,[4] A De Bel,[5] C Seguin-Devaux,[6] S De Wit,[7] L Vandekerckhove[1] and A-M Geretti[2]

[1]*AIDS Reference Laboratory and AIDS Reference Centre, Ghent University and Ghent University Hospital, Ghent, Belgium,* [2]*University College Medical School and Royal Free Hampstead National Health Service Trust, London, UK,* [3]*AIDS Reference Laboratory and Center, University of Liège, CHU de Liège, Domaine Universitaire du Sart-Tilman, Liège, Belgium,* [4]*AIDS Reference Laboratory Institute of Tropical Medicine, Antwerp, Belgium,* [5]*AIDS Reference Laboratory of the Vrije Universiteit Brussel (VUB), Subunit Universitair Ziekenhuis Brussel, Brussels, Belgium,* [6]*Laboratory of Retrovirology, CRP-Santé, Luxembourg and* [7]*Department of Infectious Diseases, Centre Hospitalier Universitaire Saint-Pierre, Brussels, Belgium*

Objective

The aim of the study was to evaluate the use of proviral DNA as a source of viral genetic material for genotypic coreceptor tropism testing (GTT).

Methods

GTT consisted of bulk V3 sequencing followed by geno2pheno interpretation with the interpretative cut-off [false positive rate (FPR)] set at 5 and 10%. GTT was performed for 165 patients with a viral load of >500 HIV-1 RNA copies/mL on simultaneously collected plasma RNA and proviral DNA, and for 126 patients with a viral load of <500 copies/mL on current proviral DNA and pretreatment plasma RNA. Phenotypic tropism testing (PTT) results were available for 142 samples.

Results

In the simultaneous RNA/DNA comparison, concordance in prediction was 95.2% (at FPR 10%) and 96.4% (at FPR 5%). Six RNA-R5/DNA-X4 and two RNA-X4/DNA-R5 discordances were observed at an FPR of 10%, and six RNA-R5/DNA-X4 discordances were observed at an FPR of 5%. In the longitudinal RNA/DNA comparison, concordance was 88.1% (at FPR 10%) and 90.5% (at FPR 5%). Eight RNA-X4/DNA-R5 and seven RNA-R5/DNA-X4 discordances were seen at an FPR of 10%, and 10 RNA-R5/DNA-X4 and two RNA-X4/DNA-R5 discordances at an FPR of 5%. The overall concordance of RNA GTT with PTT was 82% (at FPR 10%) and 83% (at FPR 5%). The overall concordance of DNA GTT with PTT was 85% (at both 10 and 5% FPRs).

Conclusions

GTT produced highly concordant tropism predictions for proviral DNA and plasma RNA. GTT on proviral DNA offers a promising approach for tropism prediction in clinical practice, particularly for the assessment of treated patients with low or suppressed viraemia.

Keywords: chemokine (C-C motif) receptor 5 (CCR5) inhibitors, genotypic tropism testing, HIV-1 coreceptor, HIV-1 proviral DNA

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Introduction

Chemokine (C-C motif) receptor 5 (CCR5) antagonists, members of the class of HIV-1 entry inhibitors, selectively inhibit the replication of CCR5-using (R5) viral strains. Before introducing a CCR5 antagonist as a component of antiretroviral therapy (ART), coreceptor usage, or viral tropism, must be determined to exclude the possibility of the presence of chemokine (C-X-C motif) receptor 4 (CXCR4)-using (X4) strains, as these are associated with poor virological response to the drug [1]. The output of the earliest HIV-1 phenotypic tropism testing (PTT) assay was the formation of syncytia in cultured MT2 cells after virus inoculation. This assay is less well suited for use in routine clinical practice because of inherent difficulties with

Correspondence: Prof. Chris Verhofstede, AIDS Reference Laboratory, University Hospital, De Pintelaan 185, B-9000 Ghent, Belgium. Tel: + 32 9 332 51 61; fax: + 32 9 332 38 41; e-mail: Chris.verhofstede@ugent.be

standardization. More recent PTT assays use recombinant viruses containing the patient-derived viral envelope to infect indicator cells that express the CD4 receptor with either the CCR5 or CXCR4 coreceptor [2,3]. Recombinant assays are reproducible, but also time-consuming, labour-intensive, technically demanding and expensive. The most broadly used recombinant PTT assay is the commercial Trofile™ developed by Monogram (San Francisco, CA, USA), which was used to screen patients in clinical trials of CCR5 antagonists. In 2008, the original Trofile™ assay (OTA) was superseded by the enhanced sensitivity Trofile™ assay (ESTA), which showed increased sensitivity for detecting CXCR4-using strains within predetermined clonal mixtures. Both OTA and ESTA require a minimal viral load of 1000 HIV-1 RNA copies/mL for reliable performance.

Genotypic tropism testing (GTT) has recently been proposed as an alternative to PTT (reviewed in [4] and [5]). GTT is based on analysis of the V3-loop sequence of the HIV-1 *envelope* (*env*) gene using bioinformatic prediction models to deduce coreceptor usage. GTT has the advantage of being less technically demanding, more rapid and less expensive than PTT, thereby meeting today's need for a fast and reliable assay for routine diagnostic practice. GTT suffers, however, from the limited sensitivity for detecting minority viral species that is intrinsic to conventional Sanger sequencing methods. As X4 or X4/R5 dual tropic (D) viruses most often occur together with R5 strains, forming mixed quasispecies (M), they may remain undetected when they represent <10–25% of the total viral population [6–8]. Despite this limitation, however, emerging data provide evidence for the reliability of bulk GTT to predict virological responses to maraviroc [9].

Both GTT and PTT rely on the initial amplification of the HIV-1 glycoprotein 160 (gp160) or gp120 coding sequence from plasma viral RNA. A minimal amount of HIV-1 RNA (>500 copies/mL) is needed for successful amplification. In many patients with early failure for whom a treatment change is considered, the HIV-1 RNA will not reach this level. In addition, for some patients a treatment change may be considered when the viral load is suppressed, for example to address problems of toxicity.

Proviral DNA may be considered a potential alternative source of viral genetic material for tropism testing in patients with low or undetectable viral load. Cellular proviral DNA contains the reservoir of archived viruses, and it has been shown that V3 sequences predicted to derive from X4 viruses are present and even enriched in this reservoir [10–12].

The aim of this study was to evaluate GTT on plasma RNA and proviral DNA for two groups of patients. The first group comprised treated and untreated patients with a viral load of >500 copies/mL who underwent parallel testing of plasma RNA and proviral DNA. For the majority of these samples, results of PTT were also available, obtained through the use of either the MT2 assay or the Trofile™ assays (Monogram). The second group comprised treated patients with a viral load of <500 copies/mL who underwent GTT on a current proviral DNA sample and on the last stored plasma RNA sample collected before the viral load dropped to undetectable levels because of ART initiation.

Patients and methods

Patients

Blood samples were collected at five AIDS Reference Centres in Belgium and Luxembourg, and at the Royal Free Hospital in London, UK.

A first series, named 'simultaneous RNA/DNA', consisted of plasma and blood cell samples collected on the same day from 220 patients with a viral load of >500 copies/mL. Of these 220 patients, 101 were treatment-naïve and 119 were treatment-experienced. Results of PTT were available for 142 individuals, after performing the MT2 assay ($n=72$), the original Trofile™ assay (OTA; Monogram) ($n=24$) or the enhanced Trofile™ assay (ESTA; Monogram) ($n=46$).

A second series of samples, named 'longitudinal RNA/DNA', was collected from 137 individuals with a viral load of <500 copies/mL. GTT was performed on a current proviral DNA sample and on a stored plasma RNA sample with a viral load of >500 copies/mL, collected shortly before starting or adapting ART. At the time of plasma sample collection, 108 patients were treatment-naïve, 20 had temporarily interrupted their ART and nine were on a failing regimen. The subtype distribution of selected samples was 67.6% B *vs.* 32.4% non-B. Samples were collected with informed consent and the study was conducted with the approval of the ethics committees of the participating institutions.

Isolation of plasma and cells

Plasma and buffy coat cells were separated from ethylenediaminetetraacetic acid (EDTA)-anti-coagulated blood and stored frozen at $-80\,^{\circ}C$ until analysis. Alternatively, peripheral blood mononuclear cells (PBMCs) were purified from the blood cell fraction by Ficoll-Hypaque density centrifugation and stored as a pellet at $-80\,^{\circ}C$ or in dimethyl sulphoxide (DMSO)-containing medium in liquid nitrogen.

RNA and DNA extraction

Viral RNA was extracted from 200 to 500 μL of plasma using the High Pure Viral RNA Kit (Roche Diagnostics Systems, Basel, Switzerland) or the Nuclisense EasyMag (BioMérieux, Durham, NC, USA). DNA was extracted from a

200-μL suspension of PBMCs or buffy coat cells with the Qiagen Whole Blood Extraction Kit (Qiagen, Hilden, Germany) or Nuclisense EasyMag. All extractions were performed according to the manufacturers' instructions.

HIV culture and MT2 assay

Ficoll-Hypaque density-purified PBMCs (10^7 cells) were used immediately after isolation for co-cultivation with 5×10^6 phytohaemagglutinin-stimulated donor PBMCs in RPMI-1640 medium supplemented with interleukin-2 as described previously [13]. Cultures were considered positive when two consecutive p24 antigen determinations revealed the presence of the viral antigen, after which the supernatant was harvested. One mL of the supernatant was transferred to a 5-mL suspension of MT2 cells [14]. Cells were checked visually for the presence of syncytia every 2 days. p24 antigen determination was performed on days 5, 10 and 20. The culture was stopped and the isolate considered MT2 negative when the p24 antigen determination was negative and syncytia remained absent at day 20.

Viral load and CD4 determination

Plasma HIV-1 RNA was quantified with the Amplicor HIV Monitor v1.5 test (Roche Diagnostics Systems), with a lower limit of detection of 50 RNA copies/mL, or the Abbott RealTime HIV-1 assay (Abbott Molecular Inc., Des Plaines, IL, USA), with a lower detection limit of 40 RNA copies/mL. The CD4 cell count was determined for the fresh blood sample by flow cytometry (using a FACScan cytofluorometer and CELLQUEST software; Beckton Dickinson, Mountain View, CA, USA). Absolute CD4 cell counts were expressed per μL of blood.

Amplification and Sanger sequencing

Amplification of a fragment spanning the V1 to V4 region of the HIV-1 *env* gene was performed using the Titan One Tube RT-PCR system (Roche), for both RNA and DNA amplification. For DNA amplification, the RT step was omitted from the thermal cycling programme. A nested polymerase chain reaction (PCR) amplification protocol was used with the outer primers 6540 (HXB2 nucleotide positions 6540–6560; forward primer) and 7701 (positions 7701–7721; reverse primer) and inner primers 6561 (positions 6561–6580; forward primer) and 7645 (positions 7645–7667; reverse primer). Sequencing reactions were run with the BigDye® Terminator Cycle Sequencing kit v. 3.1 (Applied Biosystems, Life Technologies, Carlsbad, CA, USA) and three degenerate internal primers: 5′-AGYRCAGTACAATGYACACATGG-3′ (forward primer 1), 5′-TCAACHCAAYTRCTGTTAAATGG-3′ (forward primer 2) and 5′-ATTACARTAGAAAAATTCYCCTCYAC-3′ (reverse primer). Sequencing products were visualized on the ABI3130xl automated sequencer and analysed using the sequence analysis software (Applied Biosystems) with the limit for recognizing minority variants in a nucleotide mixture set at 25%.

Before submitting the V3 sequences to the coreceptor prediction tool, all chromatograms were examined manually and, if needed, edited using the proofreading module of the SMARTGENE™ HIV software package (Integrated Database Network System, Zug, Switzerland). Results were obtained after singular testing, but RNA/DNA discordant samples were all retested starting from the nucleic acid extract.

Coreceptor tropism prediction

Tropism prediction was performed with the clonal geno2pheno (G2P) prediction algorithm (http://coreceptor.bioinf.mpi-inf.mpg.de/index.php). The reported false positive rate (FPR) was used as quantitative output. The FPR indicates the probability of classifying an R5 virus falsely as X4. For the comparison of the categorical predictions (presence or absence of CXCR4 using viruses), the cut-off FPR for discrimination between CCR5 and CXCR4 use was set at 10 and 5%.

Results of PTT were reported as positive or negative for the MT2 assay and as R5, X4, dual mixed (DM) or nonreportable (NR) for the Trofile™ assays (OTA and ESTA).

Statistical analysis

Scatter plots and correlation coefficients were used to analyse the agreement of absolute FPR results. The correlation between the categorical outcomes of different assays was assessed using Cohen's kappa statistics (MEDCALC statistical software; www.medcalc.be/).

Results

GTT on simultaneously collected plasma RNA and proviral DNA samples from patients with a plasma viral load of >500 copies/mL

Simultaneous plasma RNA and proviral DNA samples were collected from 220 patients with a viral load of >500 copies/mL. The mean viral load was 27 206 copies/mL (range 501–1 258 935 copies/mL); the mean CD4 count was 365 cells/μL (range 0–1108 cells/μL). Envelope PCR amplicons and V3 sequences were obtained for 194 plasma RNA and 198 proviral DNA samples, yielding success rates

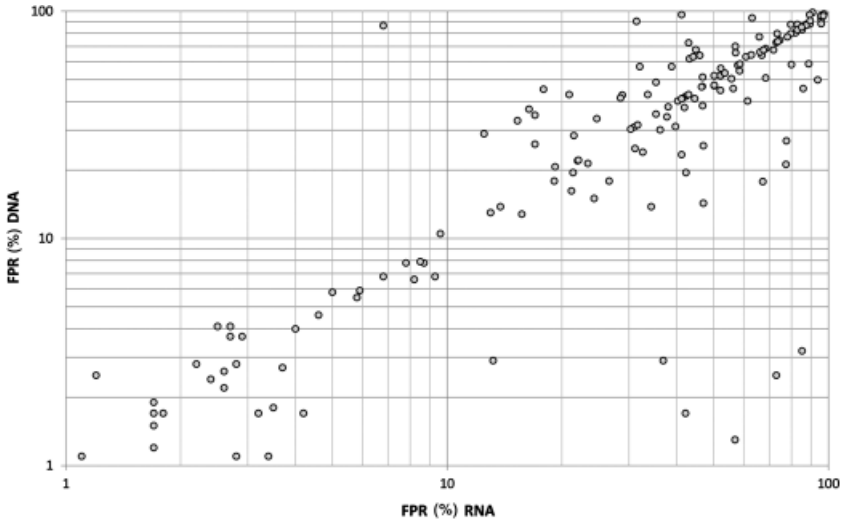

Fig. 1 Scatter plot for the comparison between the outcomes of RNA V3-based coreceptor tropism prediction (*x*-axis) and DNA V3-based prediction (*y*-axis) for 165 paired RNA/DNA samples. Predictions were performed with the geno2pheno coreceptor tool and results are expressed as false positive rate (FPR; %). In order to obtain a normal distribution the logarithm ($\log_{10}$) of the FPR was plotted. The correlation coefficient for the linear regression was $r = 0.8510$.

for amplification and sequencing of 88.2 and 86.4%, respectively. Simultaneous RNA and DNA tropism predictions were obtained for 165 patients. A scatter plot of the comparison between the G2P FPRs obtained for the plasma RNA and proviral DNA sequences is shown in Figure 1. The overall correlation coefficient (*r*) was 0.8510 [95% confidence interval (CI) 0.8026–0.8883].

Setting the FPR at 10% resulted in 38 (23.0%) plasma RNA and 42 (25.5%) proviral DNA samples predicted as X4 and an overall concordance in prediction of 95.2% ($K = 0.868$). Concordant R5 and X4 results were obtained in 121 (73.3%) and 36 (21.8%) patients, respectively. Discordant results were obtained in eight (4.8%) patients overall, comprising six RNA R5/DNA X4 discordances and two RNA X4/DNA R5 discordances (Table 1). Setting the FPR at 5% resulted in 28 (15.6%) plasma RNA and 33 (20.0%) proviral DNA samples predicted as X4 and an overall concordance in prediction of 96.4% ($K = 0.878$). Concordant R5 and X4 results were obtained in 132 (80.0%) and 27 (16.4%) patients, respectively. Discordant results were obtained in six (3.6%) patients and comprised only RNA R5/DNA X4 discordances (Table 1).

PTT results were available for 142 of 165 (86.1%) individuals. The overall concordance between GTT and PTT was >79% (Table 2), with no significant changes when setting the FPR at 10 or 5%. In comparison with MT2 and ESTA, the concordance between PTT and GTT was higher for the GTT performed on proviral DNA relative to plasma RNA, although the differences were small. In comparison with OTA, the concordance was slightly better for the prediction based on plasma RNA.

GTT with longitudinal plasma RNA and proviral DNA samples from patients with a plasma viral load of <500 copies/mL

Longitudinal RNA and DNA samples were collected from 137 individuals with a viral load of <500 copies/mL. GTT was performed on a current proviral DNA sample and on the last available stored plasma RNA sample with a viral load >500 copies/mL. The latter had been collected a maximum of 3 months before the patient started suppressive ART and the mean interval between the two sample types was 53.7 months (range 9–163 months). At the time of plasma collection, the mean CD4 count was 237 cells/μL (range 5–918 cells/μL) and the mean viral load was 47 031 copies/mL (range 1300–10^7 copies/mL). At the time of proviral DNA collection, the mean CD4 count was 616 cells/μL (range 70–1570 cells/μL), and 134 of 137 (97.8%) patients had a viral load below the quantification limit of the assay. Three had a detectable viral load (50, 125 and 141 copies/mL, respectively).

Envelope PCR amplicons and V3 sequences were obtained for 129 plasma RNA samples and 127 proviral DNA samples, yielding success rates for amplification and sequencing of 94.2 and 92.7%, respectively. Both RNA and DNA tropism predictions were available for 126 patients. A scatter plot of the FPR obtained for the two sample types is shown in Figure 2. The overall correlation coefficient (*r*) was 0.8297 (95% CI 0.7660–0.8773).

Setting the FPR at 10% resulted in 35 (27.8%) plasma RNA and 34 (27.0%) proviral DNA samples predicted as X4 and an overall concordance in prediction of 87.3% ($K = 0.701$). Concordant R5 and X4 results were obtained in

Table 1 Results and characteristics of samples with discordant coreceptor tropism predictions for plasma RNA and proviral DNA

	ID	Log viral load at RNA collection date	Subtype in Pol	Duration of viral suppression (months)	PTT	RNA FPR (%)			DNA FPR (%)			Switches in tropism prediction*	
						First result	Replicate	Replicate	First result	Replicate	Replicate	RNA/DNA single	RNA/DNA triplicate†
Paired RNA/DNA	9119	5.0	B	—	MT2 neg	36.9	36.9	36.9	2.9	2.7	2.9	R5/X4	R5/X4
	9221	5.3	B	—	MT2 pos	13.2	31.6	31.6	2.9	6.0	6.0	R5/X4	R5/X4
	947	4.9	B	—	MT2 pos	42.2	42.2	42.2	1.7	1.7	1.7	R5/X4	R5/X4
	961	5.2	B	—	MT2 pos	73.0	4.0	72.1	2.5	1.7	1.7	R5/X4	X4/X4
	8115	4.6	B	—	OTA R5	56.9	56.9	56.9	0.5	1.7	0.5	R5/X4	R5/X4
	3114	3.3	02_AG	—	ESTA D/M	85.3	85.3	85.3	3.2	4.6	4.0	R5/X4	R5/X4
	2271	2.8	02_AG	—	—	6.8	93.8	96.4	87.8	86.5	85.4	X4/R5; 10% FPR	X4/R5; 10% FPR
	12	3.8	C	—	ESTA R5	9.6	—	—	10.5	17.0	—	X4/R5; 10% FPR	X4/R5; 10% FPR
Longitudinal	9228	4.6	B	87	—	17.1	17.1	17.1	1.7	1.7	1.7	R5/X4	R5/X4
RNA/DNA	621	5.2	B	41	—	10.9	10.9	10.9	1.5	1.7	1.7	R5/X4	R5/X4
	659	5.0	B	39	—	13.4	—	—	1.8	3.4	1.8	R5/X4	R5/X4
	3	4.7	B	32	—	21.0	28.7	—	0.5	0.5	0.5	R5/X4	R5/X4
	6	3.6	B	58	—	17.0	1.8	—	1.7	1.7	0.5	R5/X4	X4/X4
	7	5.5	B	45	—	33.2	24.2	24.2	4.8	49.7	6.7	R5/X4	R5/X4
	9221	3.1	B	140	—	31.6	33.7	33.7	6.8	5.0	6.7	R5/X4; 10% FPR	R5/X4; 10% FPR
	33	4.9	B	47	—	7.8	6.0	6.6	1.7	1.7	1.7	R5/X4; 5% FPR	R5/X4; 5% FPR
	369	5.5	B	70	—	6.9	6.9	6.9	2.7	2.4	2.4	R5/X4; 5% FPR	R5/X4; 5% FPR
	415	4	B	35	—	9.0	9.0	9.0	4.0	3.7	4.1	R5/X4; 5% FPR	R5/X4; 5% FPR
	625	>5.0	01_AE	41	—	8.7	8.7	8.7	0.2	0.2	0.2	R5/X4; 5% FPR	R5/X4; 5% FPR
	2	4.4	A	43	—	4.7	4.7	4.7	77.9	—	—	X4/R5	X4/R5
	21	5.3	D	55	—	2.1	1.1	—	54.5	73.7	—	X4/R5	X4/R5
	572	5.0	G	45	—	7.8	7.8	7.8	10.5	10.5	9.0	X4/R5; 10% FPR	X4/X4; 10% FPR
	8917	4.6	B	52	—	9.9	23.6	9.9	15.6	37.4	39.1	X4/R5; 10% FPR	X4/R5; 10% FPR
	9522	5.0	B	113	—	8.5	7.4	7.4	17.9	6.8	11.5	X4/R5; 10% FPR	X4/X4; 10% FPR
	9322	5.6	A	102	—	9.3	10.2	16.6	13.7	10.2	10.2	X4/R5; 10% FPR	X4/R5; 10% FPR
	49	4.3	B	63	—	6.9	5.8	15.7	24.7	24.7	38.0	X4/R5; 10% FPR	X4/R5; 10% FPR
	24	5.2	B	40	—	9.6	5.3	9.6	30.1	79.7	—	X4/R5; 10% FPR	X4/R5; 10% FPR

ESTA, enhanced sensitivity Trofile™ assay; FPR, false positive rate; ID, identification; OTA, original Trofile™ assay; PTT, phenotypic tropism prediction; —, not available or not applicable; D/M, dual/mixed tropic.
*10% FPR or 5% FPR is added to the interpretation when the discordant result is only obtained with this specific FPR.
†Grey shading indicates that the interpretations of single and triplicate analyses are different.

84 (66.7%) and 27 (21.4%) patients, respectively. Discordant results were observed in 15 (11.9%) patients overall, comprising seven RNA R5/DNA X4 discordances and eight RNA X4/DNA R5 discordances (Table 1). Setting the FPR at 5% resulted in 20 (15.9%) plasma RNA and 28 (22.2%) proviral DNA samples predicted as X4 and an overall concordance in prediction of 90.5% ($K = 0.693$). Concordant R5 and X4 results were obtained in 96 (76.2%) and 18 (14.3%) patients, respectively. Discordant results were observed in 12 (6.9%) samples, consisting of 10 RNA R5/DNA X4 and two RNA X4/DNA R5 discordances (Table 1).

Characteristics of RNA/DNA discordances

For all samples with discordant results between plasma RNA and proviral DNA, repeat triplicate amplification and sequencing of the purified RNA and DNA were attempted. Results are summarized in Table 1. By assigning an X4 prediction to the sample whenever one of the replicate tests yielded an X4 result, the number of discordances was reduced from eight to seven for the simultaneous samples, and from 19 to 16 for the longitudinal samples. The majority of discordances (17 of 27; 63.0%) were attributable to the prediction of R5 for plasma RNA and X4 for proviral DNA. For seven of these discordant samples, PTT was performed. The PTT result confirmed the plasma RNA GTT result in two cases and the proviral DNA GTT result in five.

Discussion

Since its introduction as the first coreceptor antagonist for clinical use in HIV-1-infected patients, maraviroc has been shown to have an excellent safety profile and a favourable outcome with regard to virological responses and CD4 T-cell gain [15,16]. Given the specific antiviral activity of CCR5 antagonists, coreceptor tropism must be determined before administration. GTT provides a rapid, inexpensive and widely available approach for tropism testing. Clinical outcome studies have recently indicated that GTT is reliable for predicting virological responses to maraviroc in both treatment-experienced and treatment-naïve patients [17,18]. In these studies, GTT was applied to plasma

Table 2 Correlation between phenotypic tropism prediction (PTT) and genotypic tropism prediction (GTT), using the results of PTT as the standard, for PTT carried out using (a) the MT2 assay, (b) the original Trofile™ assay, (c) the enhanced Trofile™ assay and (d) any of these assays, for an overall comparison

		True neg	False pos	False neg	True pos	Sensitivity (%)	Specificity (%)	Concordance (%)	Kappa
GTT *vs.* MT2 ($n = 72$)									
FPR 10%	RNA	49	6	6	11	65	89	83	0.538
	DNA	48	7	3	14	82	87	86	0.644
FPR 5%	RNA	52	3	9	8	47	95	83	0.474
	DNA	52	3	6	11	65	95	88	0.631
GTT *vs.* OTA ($n = 24$)									
FPR 10%	RNA	18	3	1	2	67	86	83	0.407
	DNA	17	4	1	2	67	81	79	0.333
FPR 5%	RNA	18	3	1	2	67	86	83	0.407
	DNA	17	4	1	2	67	81	79	0.333
GTT *vs.* ESTA ($n = 46$)									
FPR 10%	RNA	32	6	3	5	63	84	80	0.407
	DNA	33	5	2	6	75	87	85	0.539
FPR 5%	RNA	34	4	4	4	50	89	83	0.395
	DNA	34	4	3	5	63	89	85	0.495
GTT *vs.* PTT (all methods) ($n = 142$)									
FPR 10%	RNA	99	15	10	18	64	87	82	0.479
	DNA	98	16	6	22	79	86	85	0.569
FPR 5%	RNA	104	10	14	14	50	91	83	0.436
	DNA	103	11	10	18	64	90	85	0.539

DNA, GTT based on V3 DNA sequences; RNA, GTT based on V3 RNA sequences; FPR, false positive rate.

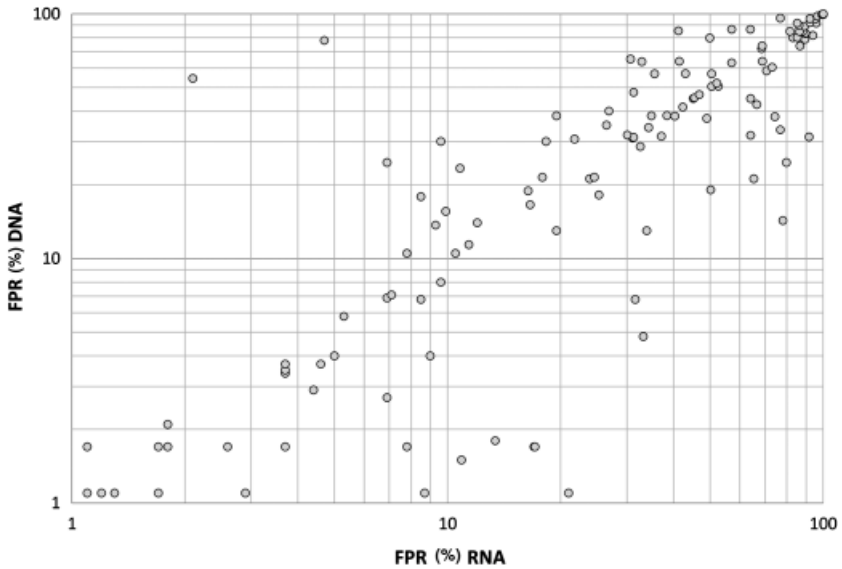

Fig. 2 Scatter plot for the comparison between the outcomes of pretreatment RNA V3-based coreceptor tropism prediction (x-axis) and on-treatment DNA V3-based prediction (y-axis), for 126 longitudinal RNA/DNA samples. Predictions were performed with the geno2pheno coreceptor tool and results are expressed as false positive rate (FPR; %). In order to obtain a normal distribution, the logarithm ($\log_{10}$) of the FPR was plotted. The correlation coefficient for linear regression was $r = 0.8297$.

samples of patients with a viral load of > 1000 copies/mL. However, there is growing interest in the possibility of using maraviroc in clinical situations other than those characterized by the presence of multidrug resistance and treatment failure, including patients with low or suppressed viraemia who may be considered for a treatment change for reasons such as toxicity or regimen simplification. In this context, proviral DNA may be considered as a source of viral genetic material for GTT.

Although evidence for a close correlation of GTT results obtained with plasma RNA and proviral DNA has previously been reported, those studies included a small number of

patients [19–21]. The aim of the present study was to explore the possibility of using proviral DNA for GTT, by comparing large series of both simultaneous plasma RNA and proviral DNA samples from patients with a viral load of >500 copies/mL, and current proviral DNA samples and stored plasma RNA samples collected from treated patients with a current viral load of <500 copies/mL.

Several algorithms for coreceptor tropism prediction from the envelope V3 sequence have been developed and evaluated [22–25]. As the aim of the study was not to compare the performances of interpretation systems, analysis was restricted to one algorithm only, geno2pheno (http://coreceptor.bioinf.mpi-inf.mpg.de/index.php), which has demonstrated comparable performance to OTA and ESTA [9]. One feature of this system is the possibility of selecting the interpretative cut-off or FPR. The higher the FPR cut-off, the greater the likelihood of detecting CXCR4-using virus, but also of falsely identifying a sequence as X4. Clinical evidence provides support for the validity of using an FPR cut-off of approximately 5–10% [17,18].

In a comparison of the results for 165 simultaneous plasma RNA/proviral DNA samples, the concordance in predicted tropism between the two sample types was high, ranging from 95.2% at an FPR of 10% to 96.4% at an FPR of 5%. In addition, the concordance in absolute FPR values for the two sample types was very good ($r = 0.8510$). Discordances were mainly attributable to X4 prediction from proviral DNA and R5 prediction from plasma RNA, thereby confirming earlier findings [12]. For four of six discordant samples, the presence of X4 strains, as detected in proviral DNA only, was supported by the results of PTT.

While the increased detection of X4 virus in proviral DNA is of interest, it should be noted that GTT and PTT by OTA or ESTA do not assess infectious virus and therefore cannot discriminate between replication-competent (and therefore clinically relevant) strains and defective strains that have no impact on virological responses to therapy. This is in contrast with the MT2 assay, which uses cultured virus. Remarkably, however, in this study the correlation between the results of the MT2 assay and GTT was higher for the proviral DNA samples (kappa coefficient 0.644 for an FPR of 5% and 0.631 for an FPR of 10%) than for the viral RNA samples (kappa coefficient 0.538 for an FPR of 5% and 0.474 for an FPR of 10%), arguing against a bias resulting from the presence of defective strains in the proviral DNA.

In a comparison of the results for 126 longitudinal plasma RNA and proviral DNA samples, the concordance in predicted tropism was 87.3% at an FPR of 10% and increased to 90.5% at an FPR of 5%. Despite an interval of a mean of 55.6 months between the two sample times, the absolute FPR values were linearly correlated ($r = 0.8297$). Moreover, in patients with long-term suppression of viraemia, the size of the proviral DNA input may be rather small, which can introduce an element of variability in the results. However, based on the results presented, the influence of this possible 'selection' bias appears to be limited. Discordant predictions were observed for 15 patients at an FPR of 10% and for 12 patients at an FPR of 5%. In contrast to the observations for the simultaneous RNA/DNA samples, changes in tropism prediction from R5 to X4 and from X4 to R5 were seen at the same frequency. Many of the changes in prediction observed with the longitudinal samples appear to reflect interpretative fluctuations around the FPR cut-off. These findings argue against a selective pressure towards X4 evolution under suppressive therapy and confirm reports from previous studies showing that changes in tropism predictions occur with low frequency in treated patients experiencing virological failure [26,27] and with even lower frequency during fully suppressive treatment, although the actual rates vary considerably from study to study [11,28,29].

The concordance between GTT and PTT varied between 79.0 and 88.0%, with kappa values varying between 0.333 and 0.644, depending on the PTT method used and the FPR chosen for GTT. These figures are comparable with previous estimates [22,23,25,29]. Although the overall concordance with PTT was higher with an FPR of 5% than with an FPR of 10%, the difference was very small.

One of the problems with both GTT and PTT is the relatively large number of failures as a result of amplification problems or problems with growing the recombinant virus or sequencing the PCR fragment. Problems with amplifying the V3 regions were anticipated for tests of proviral DNA from patients on long-term suppressive ART, as the overall load of latently infected cells is generally low in these individuals. Additionally, a high level of quasispecies variability might lead to problems in reliably interpreting the sequencing results. The overall number of amplification failures as well as sequencing failures, however, remained low, both for the plasma RNA and for the proviral DNA samples. With failure rates of 8.6% for plasma RNA and 10.4% for proviral DNA, the success ratio of GTT was higher than that reported for the Trofile™ assay, where the number of nonreportable results can be as high as 25% [30].

In conclusion, the results of this study clearly demonstrate that coreceptor tropism predictions from viral RNA and proviral DNA V3 sequences are highly comparable, both for samples collected simultaneously in viraemic patients and for longitudinal pre-ART plasma RNA and on-ART proviral DNA samples collected from patients with low or undetectable viraemia. These results suggest new possibilities for the implementation of GTT strategies in routine clinical practice, which would increase the number

of HIV-infected individuals able to benefit from treatment with a coreceptor antagonist.

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