Longitudinal analysis of HIV-1 coreceptor tropism by single and triplicate HIV-1 RNA and DNA sequencing in patients undergoing successful first-line antiretroviral therapy

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Objectives: Maraviroc has been shown to be effective in patients harbouring CCR5-tropic HIV-1. While this CCR5 antagonist has initially been used in salvage therapy, its excellent safety profile makes it ideal for antiretroviral treatment simplification strategies in patients with suppressed plasma viraemia. The aim of this study was to compare HIV-1 tropism as detected in baseline plasma RNA and peripheral blood mononuclear cell (PBMC) DNA prior to first-line therapy and to analyse tropism evolution while on successful treatment.

Methods: HIV-1 tropism was determined using triplicate genotypic testing combined with geno2pheno[coreceptor] analysis at a 10% false positive rate in 42 patients. Paired pre-treatment plasma RNA and PBMC DNA and two subsequent PBMC DNA samples (the first obtained after reaching undetectable plasma HIV-1 RNA and the second after at least 2 years of suppression of plasma viraemia) were evaluated.

Results: Coreceptor tropism was completely concordant in paired pre-treatment RNA and DNA, with 26.2% of HIV-1 sequences predicted to be non-CCR5-tropic. During follow-up, coreceptor tropism switches were detected in 4 (9.5%) patients without any preferential direction. Although false positive rate discrepancies within triplicates were common, the rate of discordance of coreceptor tropism assignment among triplicate results in this mostly CCR5-tropic dataset was only 2.1%, questioning the added value of triplicate testing compared with single testing.

Conclusions: HIV-1 coreceptor tropism changes during virologically successful first-line treatment are infrequent. HIV-1 DNA analysis may thus support the choice of a CCR5 antagonist in treatment switch strategies; however, maraviroc treatment outcome data are required to confirm this option.

Keywords: HIV type 1, V3, gp120, genotype interpretation

Introduction

Maraviroc is the only coreceptor antagonist currently licensed for treatment of HIV-1 infection.[1] Determining HIV coreceptor tropism is a prerequisite before maraviroc treatment because HIV-1 can use the CCR5 (R5 virus) or CXCR4 (X4 virus) membrane receptors, or both (DM virus), to enter target cells and maraviroc is effective only against R5 virus. While HIV-1 coreceptor tropism can be measured phenotypically by a few highly complex standardized assays, most of the screening of patient candidates for maraviroc treatment in Europe is performed by a genotypic test.[2] Genotypic tropism testing is based on the high correlation between specific sequence motifs in the HIV-1 gp120 hypervariable region 3 (V3) and coreceptor usage.

Although maraviroc use in first-line treatment regimens is acceptable under special circumstances,[3] virtually all patients

undergoing maraviroc-based therapy have previously failed anti-retroviral therapy (ART). An interesting option gaining attention is switching to maraviroc-containing regimens in patients with sup-pressed viremia.[4] The main reason for this strategy is the excellent safety profile of maraviroc, which can decrease treatment toxicity and thus possibly improve adherence.[5] However, coreceptor tropism in these patients cannot be determined using plasma HIV-1 RNA, which has been used in clinical trials. Possible alternatives include testing peripheral blood mononuclear cell (PBMC) HIV-1 DNA or the last available plasma HIV-1 RNA sample with detectable vir-aemia. Few studies[6–8] have so far addressed this issue, and only par-tially, as they analysed small or unselected patient populations and failed to use triplicate testing, which is now recommended by current European genotypic coreceptor tropism testing guidelines.[2]

In this study we compared baseline coreceptor tropism, as detected by triplicate HIV-1 plasma RNA and PBMC DNA sequen-cing before successful first-line therapy, and evaluated the evolu-tion of tropism in HIV-1 PBMC DNA while on suppressive therapy in a total of 42 patients. In addition, the use of triplicate analysis allowed us to estimate the difference in sensitivity and specificity of detection of X4/DM virus by single compared with triplicate analysis.

Methods

Plasma and PBMC samples were obtained from the Italian Cohort of Anti-retroviral Naive Patients (ICONA) biobank upon patients' informed consent. Selection criteria included achieving undetectable viraemia (defined as HIV-1 RNA <50 copies/mL) within a maximum of 32 weeks of first-line treatment and remaining on continued suppressive therapy. Paired plasma and PBMC samples were collected within 4 weeks of the start of ART (pre-ART RNA and pre-ART DNA). Two follow-up DNA samples had to be available, the first after reaching undetectable plasma HIV-1 RNA (T1 DNA) and the second after at least 2 years of continued virological suppression (T2 DNA).

Plasma RNA was extracted using the QIAamp Viral RNA Mini Kit (Qiagen, Hilden, Germany) following virus enrichment through centrifugation of 1.8 mL of plasma. PBMC DNA was extracted by using the High Pure Viral Nucleic Acid Kit (Roche, Branchburg, NJ, USA) as specified by the manufac-turer. Triplicate RT–PCR was performed by three separate RT reactions driven by random hexamers, followed by nested PCR with outer primers P150 (coordinates 6945–6971 in the HXB2 genome) and P151 (7661–7689) and inner primers P537 (6955–6976) and P538 (7353–7373). The RNA extract input volume for each RT–PCR corresponded to 0.6 mL of plasma. The same HIV-1 DNA region was subjected to triplicate amplifica-tion from PBMC DNA, representing 50 000–100 000 cells. Sequencing was performed with BigDye 3.1 chemistry using an ABI 3130xl apparatus. Elec-tropherograms were manually edited by one operator using the DNAstar Seqman Pro 7.1.0 software module and sample coding was blinded until completion of analysis of all samples.

Sequences were interpreted using the geno2pheno[coreceptor] algo-rithm (http://coreceptor.bioinf.mpi-inf.mpg.de/index.php). Primary analysis was done considering virus as R5 when all of the three replicate sequences had a false positive rate (FPR) $\geq 10\%$ and as X4/DM when at least one repli-cate sequence had an FPR $<10\%$, as recommended in current genotypic tropism testing guidelines.[2] Sensitivity, specificity, positive predictive value and negative predictive value were referred to detection of X4/DM virus. In addition, because the need for triplicate analysis in clinical practice is not widely accepted,[9] concordance between single and triplicate testing was analysed considering 10% and 20% FPR for single testing and 10% and 5.75% for triplicate testing, based on guidelines and on clinical validation of triplicate genotypic tropism testing using clinical trial data.[10,11] To determine the rate of agreement between single and triplicate analysis, the single FPR value was generated by computer-assisted random selection of one of the three triplicates and compared with the lowest FPR value obtained for the corresponding triplicate analysis. The process was repeated 1000 times and the mean $\pm$ SD was calculated.

To verify inter- and intra-patient data consistency, all the sequences obtained underwent phylogenetic analysis by the neighbour-joining method using the PHYLIP software package version 3.69 (http://evolution.genetics.washington.edu/phylip.html).

Results

Baseline characteristics of patients

Table 1 shows the characteristics of the 42 patients included in the study. Due to low sample quality or labelling inconsistencies, two pre-ART DNA samples and three follow-up samples (one T1 DNA and two T2 DNA) obtained from five different patients could not be analysed. Thus, 163 samples (42 plasma and 121 PBMC) from 42 patients were analysed. A minority of sequences [5 of 363 (1.4%) from three DNA samples, and 2 of 126 (1.6%) from two RNA samples] could not be read due to extensive overlap of mul-tiple quasispecies of different lengths and were discarded from the analysis.

The median (range) time between pre-ART and T1 DNA and between T1 DNA and T2 DNA sampling was 57 (50–94) and 120 (80–238) weeks, respectively. Using the reference 10% FPR with triplicate analysis, 11 of the 42 (26.2%) pre-ART RNA sequences were labelled as X4/DM. There was complete concordance between pre-ART RNA and pre-ART DNA tropism in all the 40 avail-able paired samples. Pre-ART X4/DM tropism was significantly asso-ciated with lower concomitant CD4 cell counts [median (range) 190 (73–384) versus 371 (294–583) cells/mm^3, $P<0.001$] but not with concomitant viraemia levels [4.32 (4.09–4.91) versus 4.71 (4.39–5.08) $\log_{10}$ copies/mL, $P=0.155$].

Table 1. Characteristics of the patients included in the study, at the time of ART initiation

Patients, n	42
Age (years), median (IQR)	38 (33–43)
Gender, % male	78.6
Heterosexual route of infection, %	48.0
Time from HIV diagnosis (weeks), median (range)	52 (2–53)
Baseline CD4 (cells/mm^3), median (range)	351 (188–520)
Baseline CD8 (cells/mm^3), median (range)	1018 (726–1297)
Baseline viral load ($\log_{10}$ HIV RNA copies/mL), median (range)	4.68 (4.30–5.01)
CDC stage C, %	7.0
Treatment, n	
2 NRTIs + 1 NNRTI	28
2 NRTIs + PI	11
other	3[a]

NRTI, nucleoside reverse transcriptase inhibitor; NNRTI, non-nucleoside reverse transcriptase inhibitor; PI, protease inhibitor.
[a]Two with 3 NRTIs and one with 3 NRTIs + 1 NNRTI.

Coreceptor tropism evolution over time

Figure 1 shows coreceptor tropism evolution over time. Tropism changes were uncommon and did not follow any preferential direction. There were 2 (4.9%) changes (both R5 to X4/DM) from pre-ART RNA to T1 DNA and 3 (7.7%) changes from T1 DNA to T2 DNA (one R5 to X4/DM and two X4/DM to R5). When comparing pre-ART RNA with T2 DNA, changes occurred in 3 (7.5%) cases (two R5 to X4/DM and one X4/DM to R5). Overall, 4 of the 42 (9.5%) patients analysed experienced at least one tropism change during the study. FPR values obtained for the V3 sequences from these patients are shown in Table 2 (FPR values for all the samples included in the study are shown in Table S1, available as Supplementary data at *JAC* Online). Of note, there was only one patient (patient 2) harbouring R5 virus at baseline and permanently switching to X4/DM during virological suppression. Phylogenetic analysis showed that for three patients the R5 and X4/DM viruses clustered together with minor divergence, while the X4/DM virus found in patient 7 only in T1 DNA was a unique population distinct from the R5 virus detected in pre-ART RNA, pre-ART DNA and T2 DNA (Figure 2). Due to the low rate of coreceptor tropism changes, it was not possible to detect any association between patients' characteristics and tropism changes over time.

Triplicate versus single sequencing analysis

FPR values were often not identical among triplicates. Of the 158 samples with triplicate results available, 114 (72.2%) were labelled as R5 and 44 (27.8%) were labelled as X4/DM based on the 10% FPR threshold. Two or three different FPR scores were generated in 43 (27.2%) and 21 (13.3%) triplicate analyses, respectively. However, only 6 (3.8%) samples, derived from five patients, showed triplicate FPR values both higher and lower than the 10% threshold and thus might have generated a false-negative R5 prediction when analysed in a single test (Table 3). This discordance rate between single analysis and the reference triplicate analysis at 10% FPR is a conservative estimate based on the unlikely worst-case scenario that the single result would be R5 in all six cases. Indeed, when comparing a 1000-fold random selection of single results with the corresponding triplicate result, the mean $\pm$ SD discordance rate obtained was $2.1 \pm 0.7\%$. Phylogenetic analysis revealed expected clustering for all of the sequences obtained from four of the five patients showing discordant triplicate results (Figure S1, available as Supplementary data at *JAC* Online). For patient 19, the three sequences obtained from pre-ART DNA formed a separate node that did not cluster with any other sequence in the whole dataset, likely reflecting
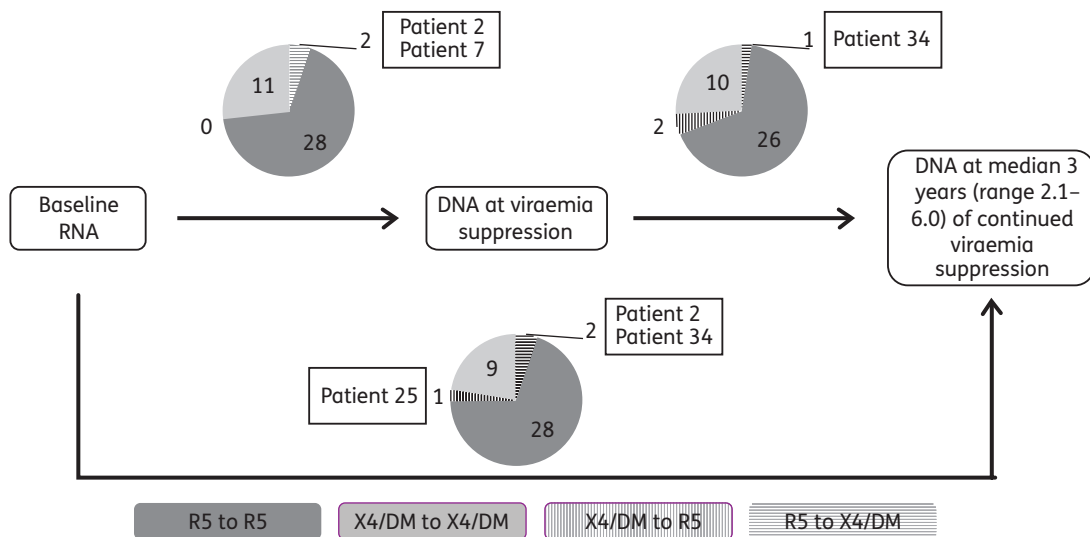

Figure 1. Evolution of HIV-1 coreceptor tropism over time in the studied patients. Boxed patient codes refer to the cases where a coreceptor tropism change occurred as detailed in Table 2.

Table 2. FPR values obtained from triplicate testing of the sample series of the four patients with changes of virus coreceptor tropism over time

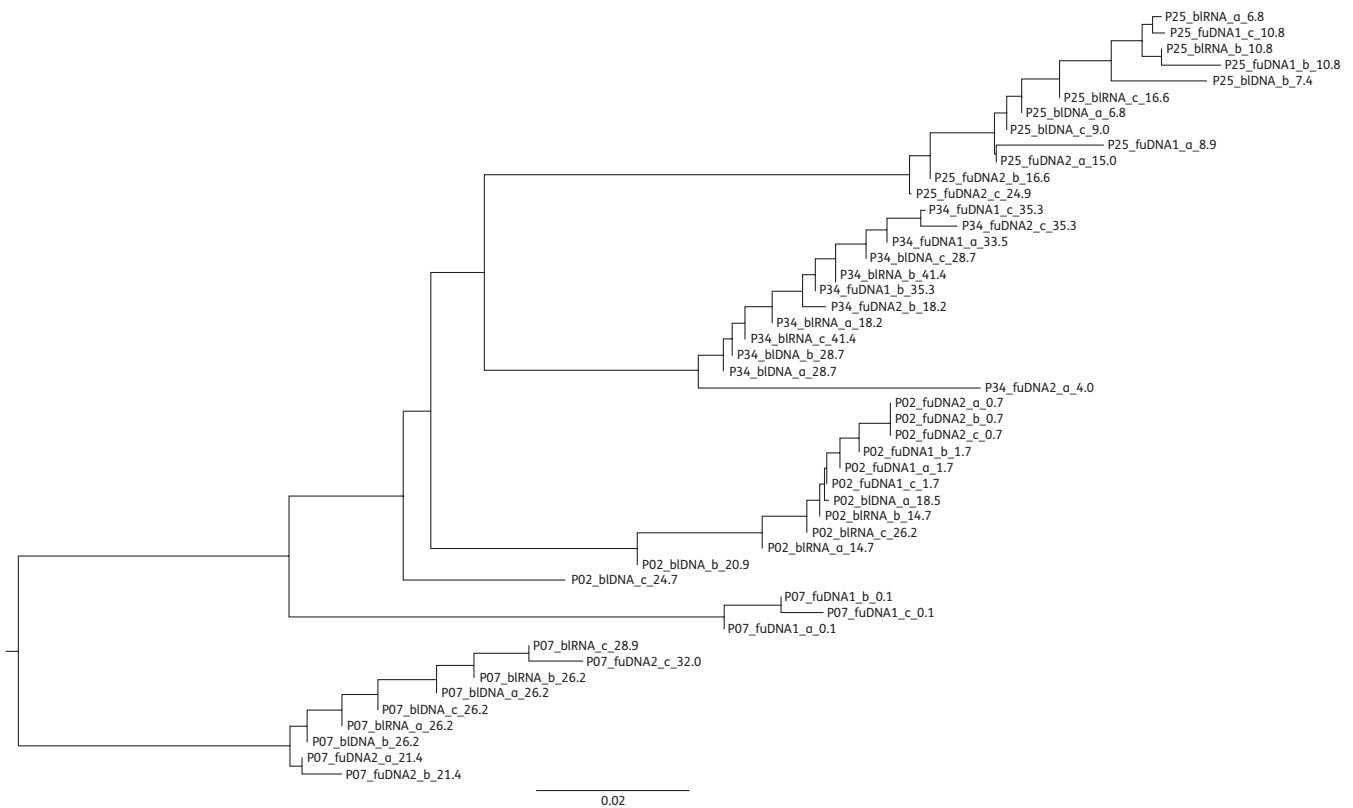| Patient ID	Triplicate pre-ART DNA FPR values			Triplicate pre-ART RNA FPR values			Triplicate T1 DNA FPR values			Triplicate T2 DNA FPR values		
2	18.5	20.9	24.7	14.7	14.7	26.2	1.7	1.7	1.7	0.7	0.7	0.7
7	26.2	26.2	26.2	26.2	26.2	28.9	0.1	0.1	0.1	32.0	21.4	21.4
25	7.4	9.0	6.8	16.6	6.8	10.8	10.8	10.8	8.9	16.6	15.0	24.9
34	28.7	28.7	28.7	18.2	41.4	41.4	33.5	35.3	35.3	35.3	18.2	4.0

Figure 2. Phylogenetic tree showing the relationships among the sequences obtained from the four patients showing coreceptor tropism switch during the study. The taxon labels indicate information in the following order: patient code, sample type (blRNA, pre-ART plasma RNA; blDNA, pre-ART PBMC DNA; fuDNA1, first follow-up PBMC DNA; fuDNA2, second follow-up PBMC DNA), replicate (a/b/c, required to distinguish sequences by the phylogenetic tree-building software), geno2pheno[coreceptor] FPR.

Table 3. FPR values obtained from triplicate testing of individual samples generating both R5 and X4/DM prediction at the 10% FPR cut-off

Patient ID	Sample type	Sequence 1	Sequence 2	Sequence 3
12	T2 DNA	28.7	1.7	1.7
19	pre-ART DNA	9.0	21.0	21.0
25	pre-ART RNA	16.6	6.8	10.8
25	T1 DNA	10.8	10.8	8.9
34	T2 DNA	35.3	18.2	4.0
37	pre-ART RNA	15.6	9.3	9.3

the existence of a distinct viral population in pre-treatment PBMC DNA.

Sensitivity, specificity, positive predictive value and negative predictive value for single versus triplicate analysis at different FPR thresholds are shown in Table 4. FPR for single analysis was evaluated at 20% and 10%. FPR for triplicate analysis was evaluated at 10% and 5.75%. Both with the 10% triplicate FPR and the 5.75% triplicate FPR taken as a reference, the increase in sensitivity when switching from 10% single FPR to 20% single FPR was counterbalanced by a much larger decrease in specificity and loss in positive predictive value. Notably, the single test using a 10% FPR threshold showed sensitivities always >90% and negative predictive values

>96.8% both in RNA and DNA samples, taking detection of X4/DM using the triplicate testing at 10% FPR as reference (the standard currently recommended by guidelines). Specificities and negative predicted values were even higher (96.5% and 98.9%, respectively), using the triplicate test with 5.75% FPR as reference (the clinically validated standard).

Discussion

While the appropriateness of genotypic coreceptor tropism screening for candidate patients for maraviroc treatment has been established based on the analysis of HIV-1 RNA,[10,11] the attractive potential of maraviroc for treatment simplification requires the analysis of HIV-1 DNA in patients with suppressed plasma HIV-1 RNA. This and two previous studies[6,8] have addressed the correlation between genotype-based tropism in HIV-1 DNA following viraemia suppression and in HIV-1 RNA before viraemia suppression.

Concordance between contemporary HIV-1 RNA and DNA tropism results obtained in treatment-naive individuals has been evaluated only in our study and the study conducted by Seclén *et al.*,[6] based on comparable numbers of cases. While we found complete concordance between paired RNA and DNA samples, Seclén *et al.*[6] reported an 18.4% discordance rate. Other studies comparing DNA and RNA genotypic coreceptor tropism in unselected patient populations have reported concordance rates

Table 4. Sensitivity, specificity, positive predictive value (PPV) and negative predictive value (NPV) for the detection of X4/DM virus based on 10% and 20% FPR single testing (10% sFPR and 20% sFPR, respectively) versus the gold standard 10% and 5.75% triplicate testing (10% tFPR and 5.75% tFPR)

Analysis	Sensitivity	Specificity	PPV	NPV
10% sFPR versus 10% tFPR (DNA and RNA)	92.28 ± 2.63	NA	NA	97.12 ± 0.96
10% sFPR versus 10% tFPR (DNA)	93.09 ± 2.71	NA	NA	97.28 ± 1.04
10% sFPR versus 10% tFPR (RNA)	90.06 ± 6.63	NA	NA	96.83 ± 2.08
20% sFPR versus 10% tFPR (DNA and RNA)	97.04 ± 1.84	81.88 ± 1.20	67.42 ± 1.52	98.63 ± 0.84
20% sFPR versus 10% tFPR (DNA)	96.03 ± 2.45	80.96 ± 1.26	67.14 ± 1.56	98.07 ± 1.18
20% sFPR versus 10% tFPR (RNA)	100.00 ± 0.00	84.39 ± 2.73	68.33 ± 3.86	100.00 ± 0.00
10% sFPR versus 5.75% tFPR (DNA and RNA)	97.15 ± 1.93	94.60 ± 0.74	83.71 ± 1.88	99.15 ± 0.57
10% sFPR versus 5.75% tFPR (DNA)	96.54 ± 2.40	94.83 ± 0.73	85.34 ± 1.78	98.88 ± 0.77
10% sFPR versus 5.75% tFPR (RNA)	100.00 ± 0.00	93.96 ± 2.03	78.27 ± 5.88	100.00 ± 0.00
20% sFPR versus 5.75% tFPR (DNA and RNA)	98.07 ± 1.89	76.39 ± 1.17	54.19 ± 1.33	99.29 ± 0.69
20% sFPR versus 5.75% tFPR (DNA)	97.60 ± 2.44	76.34 ± 1.26	56.23 ± 1.47	99.04 ± 0.97
20% sFPR versus 5.75% tFPR (RNA)	100.00 ± 0.00	76.68 ± 2.42	47.77 ± 2.64	100.00 ± 0.00

NA, not applicable (specificity and PPV are 100% by definition).
The analysis was performed on the whole DNA and RNA, and on PBMC DNA and plasma RNA separately. Values are the mean ± SD obtained from 1000 random selections of single results.

ranging from 82% to 95%.[12–16] While the small sample size may have limited the rate of disagreement between DNA- and RNA-based tropism, the increased concordance in our study can also be explained by the use of triplicate versus single testing, resulting in higher probability of detection of X4/DM virus in samples with both R5 and X4/DM populations. Also, testing patients before first-line therapy may have selected cases with a shorter duration of HIV infection compared with other studies, limiting the time of divergent evolution of coreceptor tropism in the plasma and PBMC compartments.

Tropism switch during follow-up of patients under continuous suppressive treatment appears to be relatively modest. Despite variability in the study design and a limited patient sample size, our results are comparable to those reported by Seclén et al.[6] and Soulie et al.,[8] suggesting an average 10% expected rate of genotypic tropism switch from the last measurement in plasma RNA before treatment and the follow-up test on PBMC DNA after at least 1 year of successful treatment. Different from previous reports analysing tropism only at baseline and one follow-up time-point,[6,8] our study considered two follow-up DNA samples and thus was more powered to detect tropism switch over time. A similar rate of evolution has also been reported comparing pre-therapy and post-therapy plasma RNA coreceptor tropism using the Trofile assay in patients interrupting suppressive therapy.[17] Importantly, none of these studies documented any preferential direction of tropism switch, resulting in around 5% R5 to X4/DM changes. A low level of HIV-1 replication is the likely explanation for the limited evolution of HIV-1 coreceptor tropism during suppressive therapy. However, the few cases of switch confirm that HIV-1 replication cannot be assumed to be completely suppressed in the context of undetectable viral load as measured by standard assays.

Overall, these data may support the use of PBMC DNA for genotypic coreceptor tropism determination in patients with suppressed viraemia who are candidates for maraviroc treatment, as suggested by preliminary evidence in patients achieving viral load suppression after multiple treatment lines.[18] However, our case file included only patients on successful first-line therapy, who may not be representative of the more likely use of maraviroc as simplification strategy in more experienced patients. In addition, it is important to note that our patients were not subsequently treated with maraviroc, so it was not possible to verify the predictive power of genotypic coreceptor tropism testing with PBMC DNA. A recent paper by Swenson et al.[19] suggested that PBMC DNA tropism may be slightly less predictive of virological outcome compared with plasma RNA tropism, although in the different context of viraemic patients starting maraviroc-including salvage therapy. Thus, more extensive clinical validation is still required to confirm that HIV-1 DNA coreceptor tropism can reliably guide the choice of maraviroc-containing simplification regimens. In addition, parallel testing with the reference phenotypic assay (the enhanced-sensitivity Trofile assay) is recommended when any genotypic assay is used in a clinical context.

Triplicate analysis revealed a relatively small gain in sensitivity of X4/DM virus detection compared with single-test analysis. In this dataset, the percentage of samples yielding FPR values both below and above the 10% threshold by triplicate testing was only 3.8% using a conservative estimate and ∼2% using random selection of single-test results. Previous studies have documented similar discordance rates with duplicate[20] or triplicate[21] testing versus single testing. It must be noted that the small sample size and high prevalence of R5 virus variants may have contributed to a low discordance rate. On the other hand, in R5-prevalent datasets such low discordance rates actually translate into a comparatively higher increase in the sensitivity of detection of X4/DM virus. For example, the 3.8% single versus triplicate discordance rate in our study results in reclassification from R5 to X4/DM tropism in six cases, corresponding to a 15.8% increase in detection of X4/DM virus when triplicate testing is used (from 38/158 to 44/158). This estimate is in agreement with previous studies.[20,21] The choice to increase sensitivity by replicate testing or by using a higher FPR threshold clearly comes at the expense of lower

specificity, i.e. denying maraviroc treatment to patients who would benefit from it. Interestingly, a 10% single-test FPR has been shown to be predictive of response to maraviroc in the French GenoTropism study.[22]

It must be noted that the sensitivity and specificity values reported in our study for single testing at different FPR are related to the triplicate amplification followed by population sequencing analysis. This approach is recommended by European guidelines[2] but is expected to have lower sensitivity than the clinically validated phenotypic gold standard.[23] Indeed, a proper assessment of the performance of any genotypic tropism assay can only be obtained when the reference phenotypic results are available. Overall, the most appropriate and cost-effective laboratory procedures and threshold FPR remain to be established, particularly in light of the forthcoming introduction of ultradeep sequencing protocols that allow more sensitive detection of X4/DM virus compared with population sequencing.[23] European guidelines recommend that the geno2pheno tropism testing report includes the method used (e.g. triplicate analysis), the FPR values obtained and clear advice as to whether the tropism result supports the use of a CCR5 antagonist or not. As with genotypic resistance testing for the other antiretroviral classes, genotypic tropism testing results may fall into a 'grey zone', where the clinician ultimately has to balance the uncertainty of the efficacy of a CCR5 antagonist in the context of the specific patient status.

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Supplementary data

Table S1 and Figure S1 are available as Supplementary data at *JAC* Online (http://jac.oxfordjournals.org/).

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