Use of Cellular HIV DNA to Predict Virologic Response to Maraviroc: Performance of Population-Based and Deep Sequencing

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Background. A tropism test is required before administration of the antiretroviral drug maraviroc. However, plasma RNA testing is not possible in patients with undetectable plasma viral loads. Here we assess genotypic testing of cellular human immunodeficiency virus (HIV) DNA from peripheral blood mononuclear cells (PBMCs) to predict virologic responses in treatment-experienced patients beginning maraviroc-containing regimens.

Methods. PBMC samples from 181 maraviroc recipients at study entry in MOTIVATE or A4001029 (51% R5 by original Trofile). The V3 loop was amplified in triplicate from cellular HIV DNA, and matching plasma RNA (n = 156). Sequencing was performed using standard population-based methods and next-generation deep sequencing, with tropism assessment as previously defined.

Results. Genotypic DNA-based tropism testing from the cellular compartment had 78%–81% sensitivity relative to RNA-based Trofile at the same time point. Cell-based genotypic tropism methods and plasma-based phenotypic and genotypic methods were predictive of virologic response. However, when classifications were discordant, the outcomes favored the plasma predictions over the DNA ones.

Conclusions. Genotypic determination of HIV tropism can be performed using cell-derived viral DNA, and is a predictor of virologic success on maraviroc in therapy-experienced patients. However, the PBMC compartment appears to be a suboptimal predictor compared to plasma.

Keywords. HIV coreceptor-usage/tropism; HIV proviral DNA; next-generation sequencing; maraviroc.

Recent advances in human immunodeficiency virus (HIV) treatment and curative strategies have led to the need for sensitive and accurate HIV tropism assays. The CCR5 antagonist antiretroviral drug class, including maraviroc [1] and others [2, 3], are most successful when used in patients with solely CCR5-using (R5) HIV, as determined with phenotypic [4, 5] or genotypic tropism assays [6–8]. Additionally, fledgling attempts at establishing long-term remission from HIV disease [9, 10] have been developed, such as using zinc-finger nucleases for disruption of the CCR5 gene. These are related to the successful cure by stem cell transplantation from a homozygous CCR5-delta-32 [11] donor to an HIV-infected patient [12]. These curative approaches will probably require prescreening with tropism assays to identify candidate patients with exclusively R5 HIV, as a viral population which uses CXCR4 would likely be unaffected by reducing CCR5 protein levels.

Sustained suppression of plasma viremia with advances in antiretroviral therapy improves patient outcomes [13] but precludes resistance and/or tropism testing from plasma due to low HIV copy numbers. Given the impracticalities and clinical consequences of treatment interruptions [14], tropism testing from HIV RNA in successfully treated patients is not possible. Still, some patients may wish to incorporate
maraviroc into an already successful antiretroviral regimen to manage side effects or simplify the regimen. For such patients, a more feasible approach may be tropism testing from HIV DNA. This material results from successful infection of cells by HIV [15, 16]. Tropism testing from HIV DNA involves polymerase chain reaction (PCR) amplification of a portion of the envelope gene followed by phenotypic testing in cell lines or genotypic testing by sequencing. Generally, the V3 loop of HIV gp120 is the main target of such approaches. Tropism testing from HIV DNA can allow patients to switch a component of their antiretroviral regimen to a CCR5 antagonist without an interruption to their existing treatment.

Here, we assess the performance of cell-based genotypic tropism testing approaches in a large group of patients entering 3 clinical trials of the CCR5 antagonist maraviroc. All patients were viremic at the baseline testing visit, allowing parallel testing and comparison of both plasma and cell-based tropism assays, as well as actual virological outcomes to the medication. Both population-based and deep sequencing approaches were applied in both compartments, giving a total of 4 different genotypic tropism tests. The abilities of these 4 methods to predict subsequent virologic response to maraviroc were compared with each other, and also with the phenotypic, plasma-based original Trofile assay (Trofile assay) at the same time point.

METHODS

Samples and Patient Composition

Peripheral blood mononuclear cell (PBMC) samples were obtained at baseline from 181 maraviroc recipients in the MOTIVATE-1 (N = 48), MOTIVATE-2 (N = 48), and A4001029 (N = 85) studies [1, 4]. These samples were deliberately selected to include a large proportion of non-R5 Trofile results (N = 89 [49%]). An approximate 1:1 ratio of R5 to non-R5 Trofile results meant that our analyses would not be skewed by overenrichment for CCR5-tropic samples—a criticism of past studies [17]. A total of 156 (86%) had matching tropism results from plasma available. The baseline time point was day 0 of treatment with maraviroc. All participants were antiretroviral therapy experienced and received at least 1 dose of maraviroc (once or twice daily) plus an optimized background therapy during the trials. Patients were screened and periodically tested on-treatment with the original Trofile assay in plasma. Results using the enhanced sensitivity Trofile assay (ESTA) or DNA-based Trofile assay were not available.

V3 Amplification and Sequencing

V3 amplification and sequencing were performed by the same methods as previously published by our group [7, 8]. In brief, triplicate nested reverse transcription PCR was used to amplify the V3 region from HIV-RNA in plasma. For HIV-DNA, 500 µL of PBMC samples were extracted with automated methods, followed by triplicate nested PCR targeting V3. Deep sequencing with a 454 Genome Sequencer FLX was performed using the second-round PCR products, which had multiplex tags, allowing 48 samples to be sequenced in each direction per run. The median read depth obtained were 2799 reads per DNA sample (interquartile range [IQR], 2057–3623) and 2088 (IQR, 1783–2579) reads per RNA sample. A description of the data processing pipeline for deep sequencing is included as Supplementary Material.

In addition, a second round PCR amplification was also performed using the same triplicate amplified template. These PCR products underwent standard, population-based sequencing on an ABI 3730 XL DNA analyzer according to previously described methods [6].

Tropism Prediction

The geno2pheno algorithm generates a false positive rate (FPR) for each input sequence [18]. Those scoring above a certain pre-selected cutoff are classified as R5. The FPR cutoff for geno2pheno tropism assignments was set previously [6, 7, 19]. Optimization of these cutoffs was performed using a random 75% of plasma screening samples from the maraviroc treatment-experienced trials, and were validated on the remaining 25%. Cutoffs were chosen that best distinguished between early response and nonresponse to maraviroc at week 8 of treatment. The maximum percentage of non-R5 variants allowed for a sample to be classified as R5 was also optimized and validated in a similar manner [20]. A sample was considered R5 if the lowest of 3 population-based V3 sequences had a geno2pheno FPR cutoff greater than 5.75 [6]. For deep sequencing, a sample was considered R5 if <2% of the variants detected fell below an FPR of 3.5 [7]. Population-based sequencing required a higher FPR cutoff than deep sequencing, likely due to the reduced sensitivity of population-based sequencing to detect minority variants. There was an additional exploration of alternative geno2pheno cutoffs in the current study.

Ethics Statement

Written informed consent was obtained from all individuals, including consent to allow other tropism testing to be performed on their samples. The University of British Columbia–Providence Health Care Research Ethics Board reviewed the research project and granted ethical approval. All data were analyzed anonymously.

Data Analysis

Patients were grouped according to the R5 or non-R5 result by each tropism assay. Concordance was calculated from the number of samples with identical tropism calls by any 2
The period from baseline to week 24 was examined for all patients. Each assay was assessed in its ability to predict responses to maraviroc plus optimized background therapy. Patients classified as having R5 HIV would be expected to have larger virologic responses to maraviroc compared to patients classified as having non-R5 HIV. Within compartments (plasma or PBMCs), data were restricted to samples with results by all available assays; this was 181 PBMC samples and 156 plasma samples. When the 2 compartments were compared, analyses were restricted to the 156 samples with results by all 5 assays. Tests for statistical significance included the Mann-Whitney U test for comparisons of median plasma viral load declines on maraviroc, the Fisher exact test for comparisons of the proportion of patients achieving virologic responses at weeks 8 and 24, and the log-rank test for differences in median time to change in phenotypic tropism.

RESULTS

Prediction of Virologic Efficacy and Tropism Changes on Maraviroc

Matched plasma and PBMC baseline samples were assessed by 2 genotypic methods and the original Trofile assay in plasma, giving a total of 5 tropism assays to compare. The short- and long-term virologic efficacy of maraviroc in patients were assessed as primary analyses. Patients were correctly classified as having R5 HIV if they were virologic responders to maraviroc-based therapy. Patients were stratified by whether they had R5 or non-R5 results by each of the 5 assays at baseline. Generally, these 5 tropism methods were all similarly predictive of virologic responses to the study medication, regardless of the specific approach or compartment (Figure 1). Short-term virologic responses by week 8 were examined, as in previous studies of deep sequencing by our group [7]. A response to maraviroc-based therapy was defined as a plasma viral load decline ≥1 log copies/mL from baseline to week 8, or having an undetectable viral load at week 8. Odds ratios of success for groups identified as R5 vs non-R5 ranged from 3.2 for deep sequencing in PBMCs to 9.4 for deep sequencing in plasma (Table 1).

At week 24, the percentage of patients with undetectable viremia ranged from 42% to 47% among the R5 groups, which was 16%–22% higher than the non-R5 groups. By all assays, patients with R5 HIV had approximately 2 times the odds of virologic suppression at week 24 compared to those with non-R5. Virologic success of R5 groups was statistically significantly higher than non-R5 groups by all assays at week 8. These groups also had significantly different virologic suppression by week 24, as classified by all assays (P < .05), except the Trofile assay (which had a trend toward significance) (Table 1). These analyses were reexamined in a subset of 81 patients who had compromised treatment background activity, and for whom maraviroc would be expected to have the largest impact. This approach can best distinguish differences...
in assay performance by minimizing the contribution of background antiretroviral agents to the activity of maraviroc. These patients, who had a weighted optimized background therapy sensitivity score $\leq 1$, had similar responses to the overall study group. In fact, the difference between the R5 and non-R5 groups was exaggerated in this subset. The patients classified as having R5 HIV by any of the tropism assays had much higher responses to maraviroc than did those with non-R5 classifications (Supplementary Table 1).

Virologic failure on maraviroc is often accompanied by a "change" in tropism from R5 to non-R5 [21]. Changes in phenotypic tropism by Trofile were also examined over the course of the trial. Analyses were restricted to those patients who had R5 Trofile results at both screening and baseline, leaving a total of 84 patients with DNA results and 71 with RNA results. Patients with non-R5 results by the genotypic assays were significantly more likely to have subsequent Dual/Mixed or X4 results by Trofile in plasma over the course of the study (Figure 2).

### Diagnostic Performance of Tropism Assays

The performance of all 4 sequencing approaches were compared against the phenotypic original Trofile assay because ESTA results were not available for these samples. Concordance of DNA-based tropism testing in cells was assessed relative to tropism classifications by the Trofile assay in the matching plasma sample. Genotypic DNA testing by population-based sequencing had 80% concordance with Trofile; DNA-based deep sequencing had 77% concordance. The corresponding RNA-based approaches had concordance with Trofile of 86%, respectively. The sensitivity, specificity, and concordance of all approaches are shown in Supplementary Table 2.

Since plasma approaches have been thoroughly characterized in the literature, the ability of the DNA-based approaches were compared against their corresponding RNA-based approaches. Relative to RNA, population-based and deep sequencing from plasma and PBMCs were 78% using population-based sequencing and 83% using deep sequencing.

### Compartmental Differences

To address the possibility that the bioinformatic algorithm could overestimate the contribution of background antiretroviral agents to the activity of maraviroc in their background regimen, additional geno2pheno cutoffs were evaluated in a group of 99 patients with a weighted optimized background therapy susceptibility score $\leq 1$.
This analysis again confirmed that a cutoff in the range of approximately 5.75–10 was able to distinguish the largest difference in week 8 plasma viral load declines between tropism groups (Supplementary Figure 1). Thus, the poorer performance of tropism testing in the cellular compartment was likely not an issue with bioinformatic cutoffs having been optimized in a plasma-based context.

For each sequencing approach, the relative performance of the plasma or PBMC predictions was assessed. Importantly, for all measures of virologic response explored, the plasma

Figure 2. All genotypic tropism testing methods predicted future phenotypic tropism change on maraviroc. Kaplan-Meier plots show the percentage of patients who change phenotypic tropism status by Trofile over the course of the study. Analysis is restricted to patients with R5 Trofile results at both screening and baseline. Patients are stratified by their baseline tropism results by each of the 4 genotypic assays, with the R5 groups shown in solid black lines and the non-R5 groups shown in dashed lines. A and B, Deep sequencing results in the peripheral blood mononuclear cell and plasma compartments, respectively. C and D, Population-based sequencing results. The P values by the log-rank test for the differences in median time to tropism change between groups were all <.05. Ticks represent censored observations. Abbreviation: pVL, plasma viral load.
compartment consistently outperformed the PBMC compartment in its predictability (Table 1). For both deep and population-based sequencing, patients identified as having R5 HIV in both compartments had virologic declines on maraviroc of approximately 2.5 logs by week 24, whereas those where both compartments indicated non-R5 HIV had declines of approximately 1 log (Figure 3A and 3B). Patients with discordant results between compartments had overall virologic responses that seemed to favor the RNA rather than the DNA predictions. At week 24, the median pVL decline was approximately 1.5 log where plasma indicated R5 but PBMCs indicated non-R5, while the median decline was approximately 0.5 log where plasma indicated non-R5 but PBMCs indicated R5 HIV. This suggests that testing from the plasma compartment was able to correctly identify more patients as maraviroc responders and nonresponders than testing from the cellular compartment.

**DISCUSSION**

This study examined the performance of tropism classifications from the cellular compartment in >150 patients starting maraviroc-based therapy. Two independent sequencing approaches from both the plasma and PBMC compartments were tested against each other and against the plasma-based phenotypic original Trofile assay. While performance was moderate from PBMCs, tropism predictions from the plasma compartment consistently outperformed the DNA-based methods. Where results were discordant, longer-term virologic suppression was poorly predicted by DNA-based methods, suggesting a level of misclassification by DNA approaches vs RNA ones. Nevertheless, this approach may be the only option for some patients, barring deliberate but inadvisable treatment interruptions [22] to raise viremia to levels needed for RNA-based tropism methods. Additionally, the reasonably high concordance (approximately 80%) between the plasma and cellular compartments should give some confidence that DNA-based approaches give useful clinical information. The use of DNA-based tropism testing is suggested in European guidelines [23], but better guidance will likely result from an ongoing clinical trial of DNA-based tropism testing in patients with suppressed viremia [24].

This study confirms and expands on the recent results from Vitiello and colleagues, who examined a group of 20 patients switching a component of their antiretroviral regimens to maraviroc while virologically suppressed [25]. Both studies found that DNA tropism testing could be used to predict successful treatment with maraviroc, arguably the most clinically relevant outcome of a tropism test. Compared to past reports, this study found roughly similar, if slightly worse, diagnostic accuracy of cell-based genotypic tropism assays relative to phenotypic and plasma-based approaches [26–29].

![Figure 3.](https://example.com/figure3.png) Patients with discordant tropism results between compartments had virologic responses that favored the plasma prediction. A, Deep sequencing results. B, Population-based sequencing results. Solid black lines indicate the groups where RNA and DNA methods both indicated R5. Solid gray lines indicate the groups where RNA and DNA both indicated non-R5. Discordant groups are indicated by dashed (RNA R5 and DNA non-R5), and dotted (RNA non-R5 and DNA R5) lines.
virologic response to maraviroc in a real clinical setting. The ability to predict these responses acted as an independent, objective confirmation of the diagnostic performance of these assays. An additional advantage of this study is the fact that paired plasma and PBMC fractions from the same blood draws could be compared for their ability to predict virologic response, whereas other studies have tended to compare later PBMC results with earlier presuppression plasma results [27, 30, 31]. This study also has the advantage of the design of the clinical trials from which these patients were drawn. The inclusion of patients enrolled in A4001029 who had non-R5 results at baseline but were still prescribed maraviroc gives additional confirmation on the utility of these methods.

There are some difficulties inherent to tropism testing from HIV DNA. The cellular “buffy coat” fraction of whole blood is not routinely collected or stored, nor are PBMCs routinely separated for analysis. The cellular compartment has also been found to have higher sequence variation [32] and higher prevalence of CXCR4-tropic HIV [33] compared to the plasma. Importantly, overestimation of CXCR4-usage may actually increase the likelihood of success with CCR5 antagonists, since more patients may be screened out by DNA-based approaches. However, our results are not definitive in their support for this hypothesis, and a prospective clinical trial is much more clinical experience. However, the DNA-based tropism testing has yet to be completed. Low input copy number may also be an issue for testing from the cellular compartment compared to the plasma [34]. Quantitation of HIV DNA and cell number were not performed in this study, so potentially low copy numbers may have contributed to performance issues. However, routine quantitation of cells or DNA copies represents a fairly significant barrier to the implementation, availability, and turn-around time of DNA-based tropism testing. Input copy number may be accounted for by the use of PCR “tags” accompanying each DNA strand amplified, as recent work has shown [35]. However, this technique was not available at the time of testing.

A major strength of this study was having access to paired plasma and PBMC samples from the same time point, which enabled comparisons between compartments. Although these patients were viremic, this sample set is ideal for comparing DNA-based approaches to RNA-based ones, for which there is much more clinical experience. However, the fact that patients did not have suppressed viremia at baseline requires that we extrapolate these results to patients with undetectable viral loads who may switch to maraviroc, which is ultimately the target group for DNA-based tropism approaches. For example, cellular HIV DNA copy number may decay with effective antiretroviral therapy [36, 37], as lower replication may decrease the pool of HIV DNA. However, this issue could not be addressed with this sample set because all patients had detectable viral loads.

The relatively small number of patients who had tropism classifications that were discordant between compartments (eg, R5 in plasma but non-R5 in PBMCs) meant it was not possible to definitively state that plasma predicts maraviroc response better than the cellular compartment. Conversely, the small number of patients with discordant results also reflects the reassuring fact that a large majority of patients in fact had concordant results between the compartments. Another potential limitation of this study is the fact that background therapy also affects response to maraviroc in addition to HIV tropism [38]. However, this would presumably affect all assays equally in their ability to predict virologic response to maraviroc-based therapy, so it should not have greatly skewed the results.

Despite the above-mentioned caveats, this study demonstrates promising potential for DNA-based tropism methods. That the cell-based classifications were not as clinically predictive as plasma-based ones should add a measure of caution to the routine use of this approach. However, the DNA-based testing was still able to discriminate between responders and nonresponders to maraviroc-containing regimens, and despite some shortcomings, may be the best course of action prior to prescribing maraviroc in patients with suppressed viremia.

Supplementary Data

Supplementary materials are available online at Clinical Infectious Diseases (http://cid.oxfordjournals.org/). Supplementary materials consist of data provided by the author that are published to benefit the reader. The posted materials are not copyedited. The contents of all supplementary data are the sole responsibility of the authors. Questions or messages regarding errors should be addressed to the author.

Notes

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Potential conflicts of interest. P. R. H. has been a paid consultant to ViiV/Pfizer. D. C., S. P., M. L., I. J., J. H., and H. V. are employees of ViiV/Pfizer.

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