Deep-Sequencing Analysis of the Dynamics of HIV-1 Quasiespecies in Naive Patients during a Short Exposure to Maraviroc

Almudena Cascajero,^a Alberto Rastrojo,^b Francisco Díez-Fuertes,^a Beatriz Hernández-Novoa,^{c*} Begoña Aguado,^b Santiago Moreno,^c José Alcami,^a Mayte Pérez-Olmeda^a

^aAIDS Immunopathology Unit, Instituto de Salud Carlos III, Centro Nacional de Microbiología, Madrid, Spain

^bCentro de Biología Molecular Severo Ochoa, Universidad Autónoma de Madrid, Madrid, Spain

^cServicio de Enfermedades Infecciosas, Hospital Universitario Ramón y Cajal, Universidad de Alcalá de Henares, Instituto Ramón y Cajal de Investigación Sanitaria, Madrid, Spain

ABSTRACT In this study, we have characterized quasiespecies dynamics and the evolution of viral tropism in naive HIV-1-infected patients treated with a short course of maraviroc monotherapy (ClinicalTrials.gov registration no. NCT01060618) independently of the tropism of the infecting virus. We randomly selected 20 patients infected with viruses displaying different basal tropisms—10 carrying R5 and 10 carrying dual/mixed X4 (DM/X4) viruses—at recruitment as determined by phenotypic assay (Trofile). Evolution of viral quasiespecies at the end of treatment was determined by ultradeep sequencing of the V3 region using a 454 Life Sciences Platform and geno2pheno (g2p) algorithm for viral tropism prediction. The false-positive rate (FPR) that defines the probability of classifying an R5 virus falsely as X4 was set at 10%. X4-specific HIV-1 viral load (VL) was calculated from sequences with an FPR of <3.75%. Virological response as defined as >1-log₁₀ copies/ml reduction in VL was detected in 70% of patients independently of the basal tropism of the infecting virus. Viral tropism remained stable, and nonsignificant differences in FPR values before and after treatment were found for the majority of patients in both tropism groups. Only three patients (one with R5 and two with DM/X4 viruses) showed an increased (>1 log) X4 VL, and one patient harboring a DM/X4-tropic virus displayed a significant reduction in FPR values at the end of treatment. Fast changes in the composition of viral populations were observed in all patients after 10 days of maraviroc (MVC) monotherapy treatment, and a complete replacement of viral quasiespecies was found in 3/10 patients carrying R5-using viruses and 4/10 patients carrying DM/X4-using viruses.

IMPORTANCE Initiation of treatment with maraviroc requires previous determination of viral tropism by genotypic or phenotypic methods because of the risk of treatment failure and selection of DM/X4-tropic variants. In this study, we confirm previous work showing that the virologic response to maraviroc is independent of basal tropism. By deep-sequencing analysis, we determined that fast changes in viral populations were due to the emergence of minority variants in some patients whereas in others generation of new strains was detected. The risk of DM/X4 selection was very low as FPR values remained stable, and only one patient showed a detrimental switch to DM/X4 variants. Our data show that some DM/X4 viruses are sensitive to maraviroc treatment probably because only a low proportion of DM/X4 viruses use preferentially the X4 receptor and contain authentically maraviroc-resistant viruses that are not accurately detected by current assays.

KEYWORDS CCR5, CCR5 antagonists, CXCR4, DM/X4 tropism, HIV-1, R5 tropism, maraviroc, tropism

Received 8 March 2018 Accepted 9 March 2018

Accepted manuscript posted online 21 March 2018

Citation Cascajero A, Rastrojo A, Díez-Fuertes F, Hernández-Novoa B, Aguado B, Moreno S, Alcami J, Pérez-Olmeda M. 2018. Deep-sequencing analysis of the dynamics of HIV-1 quasiespecies in naive patients during a short exposure to maraviroc. *J Virol* 92:e00390-18. <https://doi.org/10.1128/JVI.00390-18>.

Editor Guido Silvestri, Emory University

Copyright © 2018 American Society for Microbiology. All Rights Reserved.

Address correspondence to José Alcami, ppalcami@isciii.es, or Mayte Pérez-Olmeda, mayteperez@isciii.es.

* Present address: Beatriz Hernández-Novoa, Medical Affairs Manager, ViV Healthcare, Madrid, Spain.

Entry of human immunodeficiency virus type 1 (HIV-1) into target cells is dependent on the binding of the envelope glycoprotein to the CD4 receptor and to one or both chemokine receptors, CCR5 and/or CXCR4 (1, 2, 3). Accordingly HIV-1 isolates are classified as R5 tropic or X4 tropic (using only the CCR5 or CXCR4, respectively), and dual/mixed (DM) (using both coreceptors) (4). In the majority of patients, R5 variants predominate during the early stage of infection while DM/X4-tropic viruses are usually found in advanced stages (5, 6, 7, 8) and are associated with higher viral load (VL), a rapid decrease in CD4⁺ cell counts, and faster clinical progression to AIDS (5, 9). The prevalence of R5-, X4-, and DM/X4-using viruses differs according to patient characteristics. In treatment-naïve patients, the prevalence of R5-using viruses is 80 to 90% whereas it is 50 to 55% in treatment-experienced patients (5, 10, 11, 12). Maraviroc ([MVC] Celsentri outside the United States; ViiV Healthcare, United Kingdom) is the first licensed drug of a class of HIV-1 inhibitors called CCR5 antagonists (13). This drug was approved for treatment-experienced adult patients infected with only R5-tropic detectable HIV-1 (14, 15). Determination of viral tropism prior to initiation with MVC is absolutely required and can be assessed by phenotypic and genotypic assays (16, 17). Genotypic assays, such as standard genotyping or deep sequencing, are based on the sequence of the third variable (V3) loop of the HIV-1 gp120. Several bioinformatics methods have been proposed over the years and are available as online tools (18–19); the geno2pheno (g2p) algorithm is the one most frequently used.

Standard genotypic methods have low sensitivity to detect minority variants below 15 to 20% of the viral population. Deep sequencing increases the sensitivity of detection of minority variants of CCR5- and CXCR4-tropic viruses. The emergence of DM/X4-using viruses during MVC therapy could be attributable to outgrowth of preexisting minority variants or to the generation of resistant viruses, leading to treatment failure (20). We have previously shown that response to a short course of MVC monotherapy was independent of viral tropism in treatment-naïve patients. A Trofile report highlighted the fact that a majority of X4-tropic variants were detected at a low level, suggesting that none was a pure X4-tropic virus, and so the Trofile assay cannot be used as a marker of viral tropism in this population (21). The main goal of the present study was to characterize the dynamics of quasispecies variation in a subgroup of HIV-1-naïve patients treated with a short course of MVC monotherapy by using ultradeep V3 pyrosequencing (454-UDS) in combination with coreceptor prediction tools (g2p). We examined populations emerging after treatment in order to identify potential switching from CCR5 to CXCR4 or rapid selection of minority preexisting CXCR4-tropic variants.

RESULTS

Study population and samples. The clinical characteristics of patients recruited in the NCT01060618 trial have been previously described (21). Briefly, the median age of selected patients was 37 years (range, 23 to 53 years), 85% were males, and in 90% HIV-1 transmission was produced by sexual intercourse. Mean baseline HIV-1 VL was 4.3 log₁₀ copies/ml. All of the patients were treatment naïve and were treated with a daily 300-mg dose of MVC monotherapy for 10 days.

Viral tropism (Trofile) and virological response. Viral tropism at baseline and at the end of treatment was determined by a Trofile assay (Monogram Biosciences). At baseline, 10 patients carried virus displaying R5 tropism, and the same numbers of patients were infected by DM/X4-using virus (Table 1). In three patients (one with R5- and two with DM/X4-using virus) the result was nonreportable (NR) at the end of treatment, which is a common result for this technique in plasma samples with a VL of ≤1,000 copies/ml. A change in HIV tropism according to the Trofile test was observed in one patient initially infected with an R5-tropic strain and in two individuals with DM/X4-tropic virus infections (Table 1). However, in these three patients a significant decrease in VL (>1 log₁₀ copies/ml) was produced by a short course of MVC monotherapy treatment (Fig. 1).

Virological response as defined as a reduction of >1 log₁₀ copies/ml in VL was detected in 80% and 60% of patients with R5- and DM/X4-tropic viruses, respectively. Median baseline and end-treatment VL were 16,157 copies/ml (range, 2,169 to 345,414

TABLE 1 Comparison of phenotypic and genotypic tropism as determined by Trofile and ultra-deep sequencing interpreted by geno2pheno^a

Patient	Baseline tropism		End-treatment tropism	
	Phenotypic (Trofile)	Genotypic (UDPS+g2p) ^b	Phenotypic (Trofile)	Genotypic (UDPS+g2p)
R5-1	R5	DM/X4	R5	R5
R5-2	R5	R5	R5	R5
R5-3	R5	R5	R5	R5
R5-4	R5	R5	R5	R5
R5-5	R5	R5	R5	R5
R5-6	R5	R5	R5	DM/X4
R5-7	R5	R5	R5	R5
R5-8	R5	DM/X4	NR ^c	DM/X4
R5-9	R5	R5	R5	R5
R5-10	R5	R5	DM/X4	R5
DM/X4_1	DM/X4	DM/X4	R5	R5
DM/X4_2	DM/X4	R5	DM/X4	R5
DM/X4_3	DM/X4	R5	NR ^c	DM/X4
DM/X4_4	DM/X4	R5	NR ^c	R5
DM/X4_5	DM/X4	DM/X4	DM/X4	R5
DM/X4_6	DM/X4	DM/X4	R5	R5
DM/X4_7	DM/X4	R5	DM/X4	R5
DM/X4_8	DM/X4	DM/X4	DM/X4	R5
DM/X4_9	DM/X4	DM/X4	DM/X4	R5
DM/X4_10	DM/X4	DM/X4	DM/X4	DM/X4

^aAnalysis was performed on patient samples before (baseline) and 10 days after maraviroc treatment (end treatment). Genotypic tropism was analyzed in sequences obtained with a frequency above 1% by ultra-deep sequencing. For R5, the FPR is $\geq 10\%$; for DM/X4, the FPR is $\leq 9.99\%$.

^bUDPS+g2p, ultra-deep sequencing interpreted by geno2pheno.

^cNR, nonreportable (by Trofile assay).

copies/ml; 4.21 log₁₀ copies/ml) and 1,217 copies/ml (range, 150 to 38,530 copies/ml; 3.09 log₁₀ copies/ml), respectively (Fig. 1). Mean VL differences were statistically significant between basal and end-treatment samples for patients carrying either R5- or DM/X4-tropic viruses. However, no statistical difference in median VL decreases was found between patients carrying viruses displaying different tropisms.

Genotypic prediction of viral tropism and evolution of V3 sequences during MVC treatment by UDPS. In order to define the evolution of viral tropism during the 10-day MVC monotherapy treatment, the following time points were assessed by ultra-deep sequencing (UDPS): pretreatment (baseline) and end of treatment (day 10).

Overall, 20 samples were processed using the GS FLX Titanium platform, and 439,039 sequences were generated with an average length of 284 bp (range, 278 bp to 291 bp). After processing and quality control, approximately one-third of the total sequences obtained were selected for the study of viral variability. Between 3,049 and 44,547 reads of the V3 loop were generated for each sample. Only sequences that were above 1% of the overall number of reads were considered for g2p analysis (FPR, 10%), and a median value was calculated for each time point.

Concordance between genotypic prediction of HIV-1 coreceptor usage by g2p and phenotypic prediction by Trofile was evaluated. We excluded end-treatment samples not reportable by Trofile (R5-8, DM/X4-3, and DM/X4-4). The results were 76% and 53% concordant for baseline and end-treatment samples, respectively.

At baseline, in two patients infected with R5-tropic virus by Trofile, a full X4 tropism was predicted by g2p on UDPS sequences (R5-1 and R5-8) (Table 1 and Fig. 2). In these patients 100% of amplified V3 sequences were defined as X4 because their FPRs were below 10% (Table 2). In both patients a significant decrease in viral load (>1 log₁₀ copies/ml) was observed (Fig. 1). In four patients infected with DM/X4-tropic virus by Trofile, the FPRs were in the R5 range (patients DM/X4-2, -3, -4, and -7) (Fig. 2). Maraviroc treatment resulted in a significant decrease in viral load in all patients except for patient DM/X4-3 (Fig. 1).

FIG 1 Log₁₀ VL at baseline and after 10 days of maraviroc monotherapy in HIV-1-infected treatment-naïve patients infected with R5- or DM/X4-using virus.

Analysis of the evolution of the FPR under MVC pressure (end treatment, 10 days) indicated that FPRs were maintained in 75% of the patients independently of the basal tropism of the infecting virus (Fig. 2), whereas a decrease of more than 50% in the score was observed in three patients (R5-9, DM/X4-3, and DM/X4-7). The FPR dropped into the X4 range only for patient DM/X4-3 (Fig. 2).

Table 2 summarizes the dynamics of DM/X4 variants at the different time points. Half of the patients in the R5 virus group and all patients in the DM/X4 virus group displayed DM/X4 variants by g2p analysis of sequences obtained by UDPS (between 20 and 100%). In two of five samples with 100% R5 variants before drug pressure (R5-6 and R5-9), the emergence of DM/X4-using viruses was detected at the end of MVC treatment.

The result of HIV-1 quasiespecies composition analyzed in 10 patients classified in the DM/X4 virus group by Trofile showed a frequency of 100% X4-using variants by deep sequencing in half of the patient baseline samples (Table 2). In all but one of these patients (DM/X4-10) a decrease in X4 variants was found at the end of MVC treatment whereas an increase in X4-using variants from 29% to 100% was observed in only one patient (DM/X4-3) and correlated with an absence of virological response. In four patients the proportion of X4-tropic sequences was similar in baseline and end-treatment samples. Overall, MVC treatment increased the proportion of X4 sequences at the end of treatment compared to the baseline level in only one patient in the DM/X4 group (DM/X4-3) and two patients in the R5 group (R5-6 and R5-9).

X4-specific plasma VL. To assess with a different parameter the impact of MVC treatment on viral quasiespecies reported as DM/X4 by Trofile, we estimated X4-specific HIV-1 VL by multiplying the number of sequences with a frequency of >1% by an FPR value below 3.75%. Instead of an FPR of <10 to define DM/X4 variants, this more stringent cutoff was chosen because it provides unfailing discrimination between R5 and X4 sequences and because MVC clinical trials have shown a strong association with

FIG 2 Median value of FPR at baseline and at end of treatment using all sequences detected with a frequency above 1% at each sampling time.

virological outcomes. X4-specific plasma VL was detected in 14 patients (4 in the R5 and 10 in the DM/X4 groups) (Fig. 3). Decay of >0.5 log₁₀ copies/ml in X4-specific VL was found in DM/X4 group end-treatment samples (0.61, *P* < 0.001). Three patients (R5-1, DM/X4-1, and DM/X4-2) showed an increase in X4-specific plasma VL (2.24, 1.37, and 2 log₁₀ copies/ml) at end treatment (Fig. 3).

Phylogenetic analysis. The inference of the evolutionary history for each data set was studied at baseline (green in figures) and once treatment with 10-day MVC monotherapy had been completed (orange in figures). The results shown in the phylogenetic tree revealed two different models, independently of phenotypic tropism.

TABLE 2 Percent X4 tropism sequences analyzed at different time points^a

Patient	Baseline tropism		End-treatment tropism	
	Phenotypic	% X4 sequences	Phenotypic	% X4 sequences
R5-1	R5	100	R5	50
R5-2	R5	25	R5	33
R5-3	R5	20	R5	22
R5-4	R5	0	R5	0
R5-5	R5	0	R5	0
R5-6	R5	0	R5	33
R5-7	R5	0	R5	0
R5-8	R5	100	NR ^b	100
R5-9	R5	0	R5	33
R5-10	R5	60	DM/X4	50
DM/X4_1	DM/X4	100	R5	22
DM/X4_2	DM/X4	20	DM/X4	25
DM/X4_3	DM/X4	29	NR ^b	100
DM/X4_4	DM/X4	25	NR ^b	29
DM/X4_5	DM/X4	33	DM/X4	0
DM/X4_6	DM/X4	100	R5	22
DM/X4_7	DM/X4	64	DM/X4	78
DM/X4_8	DM/X4	100	DM/X4	30
DM/X4_9	DM/X4	100	DM/X4	9
DM/X4_10	DM/X4	100	DM/X4	100

^aThe Trofile assay was used for phenotyping, and 454 sequencing was used to determine the percentage of sequences showing DM/X4 tropism.
^bNR, nonreportable by Trofile assay.

FIG 3 Log₁₀ X4 VL at baseline and at end of treatment with maraviroc monotherapy in HIV-1-infected treatment-naïve patients infected with R5- or DM/X4-using virus. X4-specific HIV-1 VL was calculated with the sequences with a frequency >1% and an FPR value below 3.75.

On one hand, for 2 out of 10 patients carrying R5-tropic virus and 6 out of 10 patients carrying DM/X4-tropic virus, the phylogenetic tree showed a topology where baseline and end-treatment samples were clearly separated. An example of each viral tropism is shown in Fig. 4A and B. These phylogenetic trees are formed by variants present prior to MVC monotherapy that were not detected at end treatment and new variants that appeared after drug pressure but which were not detected before treatment. On the other hand, samples from 8 out of 10 patients carrying R5-tropic virus and 4 out of 10 patients carrying DM/X4-tropic virus yielded a phylogenetic tree with a clear intermingling of sequences from different treatment sampling time points, indicating the existence of various viral variants that persist throughout the treatment.

DISCUSSION

In this study, we provide a detailed analysis of coreceptor usage evolution during a short exposure to MVC monotherapy in naïve patients with the main purpose of identifying a potential switch from CCR5 to CXCR4 or rapid selection of minority CXCR4 preexisting strains. The appearance of DM/X4-using variants during this treatment was analyzed using V3 sequences generated by deep sequencing from plasma samples and a V3-based coreceptor prediction tool (g2p). The use of deep sequencing in this study allowed the detection of minority variants that were not reported using conventional sequencing assays.

A major strong point of this study is that naïve patients were selected from an open-label phase II clinical trial, permitting access to plasma samples from the same time point and establishing an HIV-1 evolution specific to MVC pressure.

A first finding of our study is that virologic responses were similar between tropism groups as classified by a Trofile phenotype test. The decrease in VL from baseline was >1 log₁₀ copies/ml even in the DM/X4 group. VL was not significantly modified in only 3 out of 10 patients in this group. A viral tropism switch in the Trofile results was observed in three patients. The change from R5 to DM/X4 virus observed in patient

FIG 4 Phylogenetic trees illustrating the phylogeny of the V3 region using only sequences obtained in more than 1% of the total analyzed. For each patient, the tree depicts the distribution of viral variants according to treatment time point (baseline is shown in green, and end of treatment is shown in orange). (A) Four representative similar trees from four patients corresponding to R5 and DM/X4 viral tropism groups. These phylogenetic trees show a distinct topology. Black triangles and gray circles show R5 and DM/X4 predicted tropisms, respectively. (B) Four examples of four patients whose phylogenetic trees show a combination of sequences from different treatment sampling times. If a sequence is maintained during treatment, it is shown with two colors. Black triangles and gray circles show R5 and DM/X4 predicted tropisms, respectively.

R5-10, in which 60% of basal sequences were in the DM/X4 range as determined by UDPS, suggests the selection of more X4-tropic variants during MVC treatment. A switch from DM/X4 to R5 virus was observed in two patients (DM/X4-1 and DM/X4-6) in whom FPR values increased with MVC treatment, suggesting that some DM/X4 viruses were sensitive to maraviroc, increasing the proportion of R5-tropic strains. Alternatively, the emergence of R5-resistant variants to maraviroc cannot be excluded, but the decrease in viral load favors the first hypothesis. Previous studies reported that conversion from X4 to R5 variants is observed shortly after the initiation of antiretroviral therapy, before complete suppression of plasma viremia (22, 23).

jvi.asm.org 8

In 5 out of 10 patients infected with R5-tropic variants according to the Trofile assay, DM/X4 variants were detected by UDPS in above 25% of all the sequences analyzed. We thus confirmed that UDPS is more sensitive in the detection of minor variants than phenotypic testing. In two of these patients (R5-1 and R5-8) a full discordance was found between Trofile and g2p results as predicted DM/X4-using variants were 100% by UDPS, with median g2p scores of 9 and 3.54, respectively. This discrepancy could be explained by a bias in the amplification and/or cloning of the viral quasispecies in these patients or by a preferential use of the CCR5 receptor despite the FPR. In the UDPS analysis, a large diversity of X4-tropic variants was detected, thus excluding an amplification bias using this technique. The decrease in viral load points to a susceptibility of HIV variants to MVC even if they display a DM/X4 genotype by g2p that would be related to preferential use of the CCR5 receptor. In the remaining patients (R5 group), there is a coexistence and coevolution of R5- and DM/X4-using variants. Actually, in more than half of the patients in both groups (70% R5 and 60% X4) the variants that emerge do not evolve *de novo* as a result of the drug pressure; rather, they emerge from preexisting minor viral populations present prior to monotherapy with MVC. These results suggest different susceptibilities to this drug of both R5 and DM/X4 populations that share preferential use of the CCR5 receptor.

One of the main questions in our study was to determine the significance and the clinical relevance of minority variants before and during CCR5 antagonist treatment. We show that the emergence and long-term predominance of CXCR4-using variants are not favored by MVC pressure in the majority of patients, independently of baseline tropism. In only two patients (R5-6 and DM/X4-3) were a significant switch in the proportion of X4-tropic variants and a decrease in FPR detected. This observation is clinically relevant because CXCR4 use is a prognostic factor for disease progression and a predictive trait for CCR5 antagonist failure and represents a prevalent escape pathway for treatment with CCR5 inhibitors (5–9).

The phylogenetic tree topology obtained with these naive patients provides two scenarios of viral evolution independently of baseline tropism. A first scenario shows a replacement of the viral population (R5- and DM/X4-using variants) at each time point. An explanation for this event could be that these variants are not replaced but were undetected because in our assay the depth of sequencing was not enough. Another possibility is that after a short exposure to MVC, new minority variants emerge as a result of drug pressure. A second evolutionary pattern points to the persistence of specific viral variants despite treatment, suggesting lower susceptibility to MVC in both R5- and DM/X4-using variants.

In our study, we expected to find an increase in X4-specific plasma VL as a result of an increase of DM/X4-using variants and a decrease in R5-using variants. However, a decrease of $>1 \log_{10}$ in total viral load and a decay of $>0.5 \log_{10}$ in X4-specific VL were found in the DM/X4 group at the end of treatment. Given these results, it is reasonable to assume that patients harboring DM/X4-tropic virus might benefit from treatment with an antagonist of CCR5.

One of the limitations of this study is that the use of deep sequencing was restricted to the V3 loop, but other regions of HIV-1 envelope, such as V1V2, may be important in coreceptor usage. The large volume of sequences obtained by UDPS is unfeasible for the genotypic determination of HIV tropism and for establishing clinical significance. We addressed this conflict by limiting the analysis to sequences that are above 1% of the total number of sequences obtained. The clinical relevance of very low minority variants is yet under debate. Currently, the X4 variant proportion above which an individual simply can be considered to carry X4 virus with confidence is not known. Our study suggests that variants below 1% do not have significant clinical relevance. Another limitation of this study is the lack of standardized cutoffs for the FPR value chosen for the g2p algorithm, which could affect the results. Some studies have considered an FPR of 3.5 or 5% (24, 25). In this work we have chosen an FPR of 10% because that is the cutoff considered in clinical studies.

The decrease of VL (total and X4) and the minor DM/X4-using variant observed in

both tropism groups suggest a treatment benefit for patients carrying DM/X4-tropic virus. However, our results could be related to short exposure to MVC, and we cannot rule out that prolonged treatment could generate MVC-resistant viruses which did not have much time to expand during the short exposure to that drug.

MATERIALS AND METHODS

Population study and biological samples. Samples from 20 treatment-naïve patients from an open-label phase II clinical trial (TROPISMVC, NCT01060618) conducted at Hospital Universitario Ramón y Cajal—IRYCIS and Hospital Universitario La Paz—IdiPaz (Madrid, Spain) between 2008 and 2012 were randomly selected for this substudy, keeping the same proportion of R5 and DM/X4 strains at basal level as determined by phenotypic tests. This trial has been previously published (21). Briefly, written informed consent was obtained from all individuals, the study was approved by the Ethical Committee of Hospital Universitario Ramón y Cajal—IRYCIS, and all data were analyzed anonymously. Eligible patients were aged ≥ 18 years old, had chronic HIV-1 infection, and had not received previous antiretroviral therapy (ART). After the initiation of the study, patients received open-label MVC monotherapy (300 mg daily) for 10 days. At all time points during the study, plasma VL was measured using branched DNA (bDNA) technology (Versant HIV-1 RNA 3.0 assay; Siemens Healthcare Diagnostics, Inc., Tarrytown, NY, USA), and CD4 cell counts were quantified by flow cytometry. Plasma samples before and at the end of treatment were sent to Monogram Biosciences to determine phenotypic tropism by Trofile assay. This phenotypic assay uses pseudotyped virus stocks that express patient-derived envelope proteins to infect cell lines engineered to express CCR5 or CXCR4. Patients were grouped according to the R5 or non-R5 analysis results obtained by Trofile; plasma samples from 20 patients were collected and reassessed retrospectively by deep sequencing at two time points (day 0, baseline; day 10, end treatment).

UDPS. Ultradeep sequencing (UDPS) analysis of the V3 region was performed on the 454 Life Sciences Platform (GS-FLX; Roche Applied Science). A 273-nucleotide (nt) fragment encompassing the V3 region was generated by nested PCR from an external PCR product containing the entire *env* gen (3,464 nt). Primers for nested PCR were designed including fusion primers, a key sequence to allow multiplexing, and a template-specific primer (MID) for amplifying the region of the 273-nt fragment containing the V3 *env* region. The PCR products were purified with a GFX purification PCR-DNA and Gel Band purification kit (GE Healthcare) and quantified with a Quant-iT Picogreen double-stranded DNA (dsDNA) assay kit (Invitrogen). Pooled PCR products were clonally amplified on capture beads in water-oil emulsion microreactors. A total of 700,000 HIV-1 *env*-enriched DNA beads were deposited in the wells of each process of deep sequencing using a Roche 454-FLX machine and titanium chemistry.

Raw read files (SFF files) were converted to fastq file format with `sff_extract` script (`seq_crumbs`). Samples were demultiplexed with an in-house Perl script, allowing no mismatches in the barcode sequence. Adaptors, primers, and barcode sequences were trimmed with an in-house Perl script. Only sequences including the full V3 region were considered. Then, sequences were clustered within 100% identity and counted with an in-house Perl script. Clusters with less than 1% of the reads were not considered for further analysis.

Genotypic prediction of viral tropism. The prediction of HIV coreceptor usage was carried out by the `g2p` algorithm (<http://coreceptor.geno2pheno.org/index.php>). The algorithm predicts genotypic tropism according to a database containing V3 sequences of known phenotypic tropism. This tool was applied only to those sequences detected with a frequency above 1% at each sampling time. The result is a quantitative value called the false-positive rate (FPR) that defines the probability of classifying an R5 virus falsely as X4. The FPR cutoff used for discrimination between CCR5 and CXCR4 was set at 10% in our assay (based on recommendations from the European Consensus Group on clinical management of HIV-1 tropism testing). In this cutoff FPR, a value below 9.99 is predictive for an X4 virus, and a value above 10 reflects an R5 virus.

This mean score was used to estimate the concordance between phenotypic tropism (Trofile) and genotypic tropism (`g2p`) and evolution of V3 sequences during MVC monotherapy treatment.

X4-specific plasma VL. X4-specific HIV-1 VL was calculated by multiplying the number of sequences with a frequency of $>1\%$ by an FPR value below 3.75%. This more stringent cutoff was chosen because it provides unfailing discrimination between R5 and X4 sequences, and MVC clinical trials have shown a strong association with virological outcomes.

Phylogenetic analyses. The alignment of the reads above 1% from all samples was performed with the MAFFT multiple-sequence alignment program. Phylogenetic trees were constructed through the FastTree program (open source software). The phylogeny of each data set was inferred by an approximately maximum likelihood (ML) method using FastTree software, version 2.1.7, and employing the general time reversible (GTR) substitution model with CAT approximation for among-site rate heterogeneity. The trees were visualized and edited using FigTree software (FigTree, version 1.4.0).

Statistical analysis. The main outcome measurement was the decay of the $\log_{10}$ HIV-1 VL between baseline and day 10 corresponding with end treatment. A decay of $>1 \log_{10}$ was considered clinically significant and was used as the cutoff. Comparison of $\log_{10}$ VL decay between the R5 and DM/X4 groups was done by means of the Mann-Whitney U test, with a 5% significance level. FPR values obtained by `g2p` for baseline and end-treatment samples were also compared by a Mann-Whitney U test, with a 5% significance level.

ACKNOWLEDGMENTS

We thank Olga Palao (AIDS Immunopathogenesis Unit) and A. Zaballos (Genomics Unit, Instituto de Salud Carlos III) for their secretarial and technical assistance, respectively. We also greatly appreciate our patients for their willingness to participate.

REFERENCES

- Deng H, Liu R, Ellmeier W, Choe S, Unutmaz D, Burkhart M, Di Marzio P, Marmon S, Sutton RE, Hill CM, Davis CB, Peiper SC, Schall TJ, Littman DR, Landau NR. 1996. Identification of a major co-receptor for primary isolates of HIV-1. *Nature* 381:661–666. <https://doi.org/10.1038/381661a0>.
- Dragic T, Litwin V, Allaway GP, Martin SR, Huang Y, Nagashima KA, Cayanan C, Maddon PJ, Koup RA, Moore JP, Paxton WA. 1996. HIV-1 entry into CD4⁺ cells is mediated by the chemokine receptor CC-CKR-5. *Nature* 381:667–673. <https://doi.org/10.1038/381667a0>.
- Feng Y, Broder CC, Kennedy PE et al. 1996. HIV-1 entry cofactor: functional cDNA cloning of a seven-transmembrane, G protein-coupled receptor. *Science* 272:872–877. <https://doi.org/10.1126/science.272.5263.872>.
- Berger EA, Doms RW, Fenyö EM, Korber BT, Littman DR, Moore JP, Sattentau QJ, Schuitemaker H, Sodroski J, Weiss RA. 1998. A new classification for HIV-1. *Nature* 391:240. <https://doi.org/10.1038/34571>.
- Connor RI, Sheridan KE, Ceradini D, Choe S, Landau NR. 1997. Change in coreceptor use correlates with disease progression in HIV-1-infected individuals. *J Exp Med* 185:621–628. <https://doi.org/10.1084/jem.185.4.621>.
- Regoes RR, Bonhoeffer S. 2005. The HIV coreceptor switch: a population dynamical perspective. *Trends Microbiol* 13:269–277. <https://doi.org/10.1016/j.tim.2005.04.005>.
- Shankarappa R, Margolick JB, Gange SJ, Rodrigo AG, Upchurch D, Farzadegan H, Gupta R, Rinaldo CR, Learn GH, He X, Huang XL, Mullins JL. 1999. Consistent viral evolutionary changes associated with the progression of human immunodeficiency virus type 1 infection. *J Virol* 73:10489–10502.
- Shepherd JC, Jacobson LP, Qiao W, Jamieson BD, Phair JP, Piazza P, Quinn TC, Margolick JB. 2008. Emergence and persistence of CXCR4-tropic HIV-1 in a population of men from the multicenter AIDS cohort study. *J Infect Dis* 198:1104–1112. <https://doi.org/10.1086/591623>.
- Koot M, Keet IP, Vos AH, de Goede RE, Roos MT, Coutinho RA, Miedema F, Schellekens PT, Tersmette M. 1993. Prognostic value of HIV-1 syncytium-inducing phenotype for rate of CD4⁺ cell depletion and progression to AIDS. *Ann Intern Med* 118:681–688. <https://doi.org/10.7326/0003-4819-118-9-199305010-00004>.
- Brumme ZL, Goodrich J, Mayer HB, Brumme CJ, Henrick BM, Wynhoven B, Asselin JJ, Cheung PK, Hogg RS, Montaner JS, Harrigan PR. 2005. Molecular and clinical epidemiology of CXCR4-using HIV-1 in a large population of antiretroviral-naïve individuals. *J Infect Dis* 192:466–474. <https://doi.org/10.1086/431519>.
- Melby T, Despirito M, Demasi R, Heilek-Snyder G, Greenberg ML, Graham N. 2006. HIV-1 coreceptor use in triple-class treatment-experienced patients: baseline prevalence, correlates, and relationship to enfuvirtide response. *J Infect Dis* 194:238–246. <https://doi.org/10.1086/504693>.
- Wilkin TJ, Su Z, Kuritzkes DR, Hughes M, Flexner C, Gross R, Coakley E, Greaves W, Godfrey C, Skolnik PR, Timpone J, Rodriguez B, Gulick RM. 2007. HIV type 1 chemokine coreceptor use among antiretroviral-experienced patients screened for a clinical trial of a CCR5 inhibitor: AIDS Clinical Trial Group A5211. *Clin Infect Dis* 44:591–595. <https://doi.org/10.1086/511035>.
- Dorr P, Westby M, Dobbs S, Griffin P, Irvine B, Macartney M, Mori J, Rickett G, Smith-Burchnell C, Napier C, Webster R, Armour D, Price D, Stammen B, Wood A, Perros M. 2005. Maraviroc (UK-427,857), a potent, orally bioavailable, and selective small-molecule inhibitor of chemokine receptor CCR5 with broad-spectrum anti-human immunodeficiency virus type 1 activity. *Antimicrob Agents Chemother* 49:4721–4732. <https://doi.org/10.1128/AAC.49.11.4721-4732.2005>.
- ViiV Healthcare UK, Ltd. Celsentri product information. http://www.ema.europa.eu/docs/en_GB/document_library/EPAR_-_Product_Information/human/000811/WC500022190.pdf. ViiV Healthcare UK, Ltd., Middlesex, United Kingdom.
- Gulick RM, Lalezari J, Goodrich J, Clumeck N, DeJesus E, Horban A, Nadler J, Clotet B, Karlsson A, Wohlfeiler M, Montana JB, McHale M, Sullivan J, Ridgway C, Felstead S, Dunne MW, van der Ryst E, Mayer H, MOTIVATE Study Teams. 2008. Maraviroc for previously treated patients with R5 HIV-1 infection. *N Engl J Med* 359:1429–1441. <https://doi.org/10.1056/NEJMoa0803152>.
- Pérez-Ortega M, Alami J. 2013. Determination of HIV tropism and its use in the clinical practice. *Expert Rev Anti Infect Ther* 11:1291–1302. <https://doi.org/10.1586/14787210.2013.852469>.
- Fätkenheuer G, Pozniak AL, Johnson MA, Plettenberg A, Staszewski S, Hoepelman AI, Saag MS, Goebel FD, Rockstroh JK, Dezube BJ, Jenkins TM, Medhurst C, Sullivan JF, Ridgway C, Abel S, James IT, Youle M, van der Ryst E. 2005. Efficacy of short-term monotherapy with maraviroc, a new CCR5 antagonist, in patients infected with HIV-1. *Nat Med* 11: 1170–1172. <https://doi.org/10.1038/nm1319>.
- Jensen MA, Li FS, van't Wout AB, Nickle DC, Shriner D, He HX, McLaughlin S, Shankarappa R, Margolick JB, Mullins JL. 2003. Improved coreceptor usage prediction and genotypic monitoring of R5-to-X4 transition by motif analysis of human immunodeficiency virus type 1 env V3 loop sequences. *J Virol* 77:13376–13388. <https://doi.org/10.1128/JVI.77.24.13376-13388.2003>.
- Díez-Fuertes F, Delgado E, Vega Y, Fernández-García A, Cuevas MT, Pinilla M, García V, Pérez-Álvarez L, Thomson MM. 2013. Improvement of HIV-1 coreceptor tropism prediction by employing selected nucleotide positions of the env gene in a Bayesian network classifier. *J Antimicrob Chemother* 68:1471–1485. <https://doi.org/10.1093/jac/dkt077>.
- Westby M, Lewis M, Whitcomb J, Youle M, Pozniak AL, James IT, Jenkins TM, Perros M, van der Ryst E. 2006. Emergence of CXCR4-using human immunodeficiency virus type 1 (HIV-1) variants in a minority of HIV-1-infected patients following treatment with the CCR5 antagonist maraviroc is from a pretreatment CXCR4-using virus reservoir. *J Virol* 80: 4909–4920. <https://doi.org/10.1128/JVI.80.10.4909-4920.2006>.
- Hernández-Novoa B, Madrid-Elena N, Dronda F, Pérez-Eliás MJ, Casado JL, Pérez-Molina JA, Moreno A, Estébanez M, González J, Zamora J, Moreno S. 2014. Virological response to short-course maraviroc monotherapy does not predict viral tropism in HIV-1-infected treatment-naïve patients. *J Antimicrob Chemother* 69:1916–1919. <https://doi.org/10.1093/jac/dku059>.
- Delobel P, Sandres-Sauné K, Cazabat M, Pasquier C, Marchou B, Massip P, Izopet J. 2005. R5 to X4 switch of the predominant HIV-1 population in cellular reservoirs during effective highly active antiretroviral therapy. *J Acquir Immune Defic Syndr* 38:382–392. <https://doi.org/10.1097/01.qai.0000152835.17747.47>.
- Philpott S, Weiser B, Anastos K, Kitchen CM, Robison E, Meyer WA, III, Sacks HS, Mathur-Wagh U, Brunner C, Burger H. 2001. Preferential suppression of CXCR4-specific strains of HIV-1 by antiviral therapy. *J Clin Invest* 107:431–438. <https://doi.org/10.1172/JCI11526>.
- Swenson LC, Mo T, Dong WW, Zhong X, Woods CK, Jensen MA, Thielen A, Chapman D, Lewis M, James I, Heera J, Valdez H, Harrigan PR. 2011. Deep sequencing to infer HIV-1 coreceptor usage: application to three clinical trials of maraviroc in treatment-experienced patients. *J Infect Dis* 203:237–245. <https://doi.org/10.1093/infdis/jiq030>.
- McGovern RA, Thielen A, Mo T, Dong W, Woods CK, Chapman D, Lewis M, James I, Heera J, Valdez H, Harrigan PR. 2010. Population-based V3 genotypic tropism assay: a retrospective analysis using screening samples from the A4001029 and MOTIVATE studies. *AIDS* 24:2517–2525. <https://doi.org/10.1097/QAD.0b013e32833e6cfb>.