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

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Running title: Evolution of HIV-1 tropism in naïve-patients after Maraviroc

Abstract

In this study we have characterized quasispecies dynamics and the evolution of viral tropism in naive HIV-1-infected patients treated with a short-course of maraviroc monotherapy (NCT01060618) independently of their tropism. We randomly selected 20 patients displaying different basal tropism -10 R5 and 10 DMX4- at recruitment as determined by phenotypic tests (Trofile®). Evolution of viral quasiespecies at the end of treatment was determined by ultra-deep sequencing of the V3 region using a 454 Life Sciences Platform and geno2pheno (g2p) algorithm for viral tropism prediction. False Positive Rate (FPR) that defines the probability of classifying an R5-virus falsely as X4 was set at 10% .Viral load (VL) X4 was calculated from sequences with FPR < 3.75.

Virological response as defined as >1 $\log_{10}$ reduction in VL was detected in 70% of patients independently of their basal tropism. Viral tropism remained stable and non-significant differences in FPR values before and after treatment was found for the majority of patients in both tropism groups. Only 3 patients (1 R5 and 2 DM/X4) showed an increased (>1log) VL-X4 and one patient with DM/X4 tropism 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-day of MVC monotherapy treatment and a complete replacement of viral quasiespecies was found in 3/10 patients carrying R5-using viruses and 4/10 DM/X4-using viruses.

Importance

Initiation of treatment with maraviroc requires previous determination of tropism by genotypic or phenotypic methods because the risk of treatment failure and selection of DM/X4-tropic variants. In this study we confirm previous work showing that 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 were 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 uses preferentially the X4 receptor and contain authentically maraviroc-resistant viruses that are not accurately detected by current assays.

INTRODUCTION

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 or CXCR4[1,2,3]. Accordingly HIV-1 isolates are classified as R5 tropic and X4 tropic (using only the CCR5 or CXCR4, respectively), and dual/mixed (DM) (using both co-receptors)[4]. In the majority of patients R5 variants predominate during the early stage of infection while DM/X4-tropic viruses are usually found in advances stages[5,6,7,8] and are associated with higher 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 is different according to patient's characteristics. In treatment-naive patients, the prevalence of R5-using viruses is 80-90% compared to 50-55% in treatment-experienced patients[5,10,11,12]. Maraviroc (MVC, Celsentri outside US, Viiv Healthcare, UK) 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 HIV-1 detectable[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]; geno2pheno (g2p) algorithm is the most frequently used one.

Standard genotypic methods have low sensitivity to detect minority variants below 15–20% 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 pre-existing 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. Trofile® report highlighted the fact that majority of X4-tropic variants were detected at a low level suggesting that none was pure X4-tropic virus so that 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-naive patients treated with a short course of MVC-monotherapy by using ultra deep 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 pre-existing CXCR4-tropic variants.

MATERIAL AND METHODS

Population study and biological samples

Samples from twenty treatment-naive 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 sub-study 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 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 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 assay by Trofile® assay. This phenotypic assay uses virus stocks pseudotyped 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 re-assessed retrospectively by deep sequencing at two time points (day 0 –basal- , day 10 -end treatment-)

Ultra Deep Sequencing (UDPS)

UDPS analysis of the V3 region was performed on the 454 Life Sciences Platform (GS-FLX; Roche Applied Science). A 273-nt fragment encompassing the V3 region 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 273-nt fragment containing the V3 env-region. The PCR products were purified with the GFX Purification PCR-DNA and Gel Band purification kit (G.E. Healthcare) and quantified with Quant-iT Picogreen dsDNA assay kit (Invitrogen). Pooled PCR products were clonally amplified on capture beads in water-oil-emulsion micro-reactors. A total of 700,000 HIV-1 *env* enriched-DNA beads were deposited in the wells of each process of deep sequencing with Roche 454-FLX machine using 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 a further analysis.

Genotypic prediction of viral tropism

The prediction of HIV co-receptor usage was carried out by the g2p (http://coreceptor.geno2pheno.org/index.php). The algorithm predicts genotypic tropism according to data-base 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 cut-off used for discrimination between CCR5 and CXCR4 was set at 10% in our assay. (Recommendations from the European Consensus Group

on clinical management of HIV-1 tropism testing). In this cut-off 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 >1% and a FPR value below 3.75. This more stringent cut-off was chosen because provides unfailing discrimination between R5 and X4 sequences and MVC clinical trials have showed strong association with virological outcomes.

Phylogenetic analyses

The alignment of the reads above 1% from all samples was performed with MAFFT multiple sequence alignment program. Phylogenetic trees were constructed through the FastTree program (open-source software). The phylogeny of each dataset 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 v1.4.0 program).

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. Decay >1 $\log_{10}$ was considered clinically significant and was used as the cut-off. 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 Mann–Whitney U-test, with a 5% significance level.

RESULTS

Study population and samples

The clinical characteristics of patients recruited in the NCT01060618 trial have been previously described[21]. Briefly, median age of selected patients was 37 years (range 23-53), 85% were males and in 90% HIV-1 transmission was produced by sexual intercourse. Mean baseline HIV-1 VL was 4.3 $\log_{10}$. All of them were treatment-naive patients and were treated with a 300 mg-daily 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 Trofile® assay (Monogram Biosciences®). At baseline, 10 patients displayed R5-tropism and same numbers of patients were infected by DM/X4-using virus (Table 1). In 3 patients (1 R5 and 2 DM/X4-using virus) the result was non-reportable (NR) at the end of treatment that is a common result for this technique in plasma samples with VL ≤1.000 copies/mL. A change in HIV tropism according to Trofile® test was observed in one patient initially infected with R5-tropic strain and 2 individuals with DM/X4 tropic viruses (Table 1). However, in these three patients a significant decrease in VL (>1 $\log_{10}$) was produced by short-course of MVC-monotherapy treatment (Fig. 1).

Virological response as defined as >1 $\log_{10}$ reduction in VL was detected in 80% and 60% of patients with an R5 and DM/X4-tropic viruses respectively. Median baseline and end-treatment VL were 16.157 copies/mL (range 2.169-345.414) (4,21 $\log_{10}$) and 1.217 copies/mL (range 150-38.530) (3.09 $\log_{10}$), 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 decrease was found between patients displaying different tropism.

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 UDPS: pre-treatment (baseline), and end of treatment (day 10).

Overall 20 samples were processed using GS FLX Titanium and 439.039 sequences were generated with a length average 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 co-receptor 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 concordant in a 76% and 53% for baseline and end-treatment samples respectively.

At baseline, in two patients with R5 tropism by Trofile® a full X4 tropism was predicted by g2p on UDPS sequences (R5-1, R5-8) (Table 1 and figure 2). In these patients 100% of amplified V3 sequences were defined as X4 because their FPR was below 10 (Table 2). In both patients a significant decrease in viral load (>1 $\log_{10}$) was observed (Figure 1). In four patients with DM/X4 tropism by Trofile® their FPR was in the R5 range

(patients DM/X4 2-3-4-7) (Figure 2). Maraviroc treatment resulted in a significant decrease in viral load in all patients excepting patient DM/X4-3 (Figure 1).

Regarding evolution of FPR under MVC pressure (end-treatment, 10 days) FPR were maintained in all patients independently of their basal tropism (Figure 2) whereas a decrease of more than 50% in the score was observed in three patients (R5-9, DM/X4-3 y DM/X4-7) but only in DM/X4-3 the FPR dropped into the X4 range (Figure 2).

Table 2 summarizes the dynamics of DM/X4 variants at the different time points. Half of patients in the R5-group and all patients in the DM/X4 group displayed DM/X4 variants by g2p analysis of sequences obtained by UDPS (between 20 and 100%). In 2 of 5 samples with 100% R5 variants before drug pressure (R5-6 and R5-9) an emergence of DM/X4 using viruses were detected at end-MVC treatment.

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

multiplying the number of sequences with a frequency >1% and a FPR value below 3.75. Instead of a FPR<10 to define DM/X4 variants, his more stringent cut-off was chosen because provides unfailing discrimination betwen R5 and X4 sequences and MVC clinical trials have showed strong association with virological outcomes.X4-specific plasma VL was detected in 14 patients (4 R5 and 10 DM/X4 group) (Fig. 3). A decay >0.5 Log10 X4-specific VL was found in DM/X4-group at end-treatment samples (0.61, $p<0.001$). Three patients (R5-1, DM/X4-1 and DM/X4-2) showed an increased in X4-specific plasma VL (2.24, 2, 0.7 and 1.37 log) at end-treatment (Fig.3).

Phylogenetic analysis.

The inference of the evolutionary history for each dataset was studied at baseline (green) and once treatment with 10-day MVC-monotherapy had been completed (orange). The results of phylogenetic tree revealed two different models, independently of phenotypic tropism. On one hand, 2 out of 10 R5- and 6 out of 10 DM/X4-tropism patients showed a topology where baseline and end-treatment samples are clearly separated. An example of each viral tropism is shown in Figure 4A and 4B. These phylogenetic trees are formed by variants present prior to MVC-monotherapy that were not detected at end-treatment and new variants that appear after drug pressure but which were not detected before treatment. On the other hand, 8 out of 10 R5- and 4 out of 10 DM/X4-tropism patients showed a phylogenetic tree with a clear intermingling of sequences from different treatment sampling time-points, indicating the existence of various viral variants that persist along the treatment.

DISCUSSION

In this study, we provide a detailed analysis of co-receptor usage evolution during a short exposure to MVC monotherapy in naïve-patients with the main purpose of

identifying potential switch from CCR5 to CXCR4 or rapid selection of minority CXCR4 pre-existing strains. The appearance of DM/X4-using variant during this treatment was analyzed using V3 sequences generated by deep sequencing from plasma samples and a V3-based co-receptor prediction tool (g2p). The use of deep sequencing in this study allowed the detection of minority variant that were not reported using conventional sequencing assays.

A major strong point of this study is that naive 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 of MVC pressure.

A first finding of our study is that virologic responses were similar between tropism groups as classified by phenotype Trofile® test. The decrease in VL from baseline was >1 $\log_{10}$ copies/mL even in the DM/X4 group. Only in three out of ten patients in this group VL was not significantly modified. A tropism switch in Trofile® result was observed in three patients. The change from R5 to DM/X4 observed in a patient R5-10 in which 60% of basal sequences were in the DM/X4 range as determined by UDPS suggests the selection of more tropic X4 variants during MVC treatment. A switch from DM/X4 to R5 was observed in 2 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 viraemia[22,23].

Our analyses of variants obtained by deep sequencing show a fast change in the composition of viral populations in all patients during a 10-day MVC-monotherapy. Together with VL decrease these data suggest both a quantitative and a qualitative impact of MVC treatment on the kinetics of viral quasiespecies independently of baseline viral tropism.

In 5 out of 10 patients infected with R5-tropic variants according to Trofile® DM/X4 variants were detected by UDPS above 25% of all the sequences analyzed. We thus confirmed UDPS is more sensitive in the detection of minor variants as compared to phenotypic testing. In two of these patients (R5-1 and R5-8) a full discordance was found between Trofile® and g2p as predicted DM/X4-using variants were 100% by UDPS with a median g2p score 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 to a preferential use of the CCR5 receptor despite the FPR. In the UDS 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 with a preferential use of the CCR5 receptor. In the rest of 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 pre-existing minor-viral populations present prior to monotherapy with MVC. These results suggest different susceptibility to this drug of both R5 and DM/X4 populations that share a 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 emergence and long-term predominance of CXCR4-using variants are not favored by MVC pressure in the majority of patients independently of basal tropism. In only two patients (R5-6 and DM/X4-3) a significant switch in the proportion of X4-tropic variants and a decrease in FPR were detected. This observation is clinically relevant because CXCR4-use is a prognostic factor for disease progression, 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 naïve-patients provides two scenarios of viral evolution independently of baseline tropism. A first scenario shows a replacement of viral population (R5 and DM/X4-using variant) at each time-point. An explanation to 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 short exposure to MVC new minority variants merge 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 of R5-using variants. However a decrease of > 1 $\log_{10}$ in total viral load and a decay >0.5 $\log_{10}$ X4-specific VL was found in DM/X4-group at end-treatment. Given these results, it is reasonable to assume those patients with DM/X4-tropism 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 to establish clinical significance. We addressed this conflict by limiting the analysis to sequences that are above 1% of total sequences obtained. The clinical relevance of very low minority variants is yet under debate. Currently it is not known the X4 variants proportion above which an individual simply can be called X4 with confidence. Our study suggests that variants below 1% do not have significant clinical relevance. Other limitation of this study is the lack of standardized cutoffs for the FPR value chosen for the g2p algorithm could affect the results. Some studies have considered a FPR of 3.5 or 5%[24,25], In this work we have chosen a FPR of 10% because is the cutoff considered in clinical studies.

The decrease of VL (total and X4) and minor DM/X4-using variant observed in both tropism groups suggests a treatment benefit for DM/X4-group patient. However, our results could be related to short exposure to MVC and we cannot rule out that a prolonged treatment could generate MVC-resistant viruses which did not have much time to expand during the short exposure to that drug.

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455 **Table 1**: Comparison of phenotypic and genotypic tropism as determined by Trofile®
456 and Ultra-deep sequencing interpreted by geno2pheno –g2p-) from the studied patients
457 before (baseline) and 10 days after maraviroc treatment (end-treatment). Genotypic
458 tropism was analyzed in sequences obtained with a frequency above 1% by UDS.

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	Baseline		End-treatment	
Patients	Phenotypic tropism (Trofile®)	Genotypic tropism (UDS+g2p)	Phenotypic tropism (Trofile®)	Genotypic tropism (UDS+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*	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*	DM/X4
DM/X4_4	DM/X4	R5	NR*	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

NR* (Non reportable -Trofile®-)

R5: FPR ≥10 DM/X4: FPR ≤9.99

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462 **Table 2:** Percent X4-tropism sequences analyzed to all-time point treatment.

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	Baseline		End-treatment	
Patients	Phenotypic tropism (Trofile®)	% sequences showed X4 tropism (454)	Phenotypic tropism (Trofile®)	% sequences showed X4 tropism (454)
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*	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*	100%
DM/X4_4	DM/X4	25%	NR*	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%

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Figure legends

Fig. 1 Log_{10} VL at baseline and after 10 days of maraviroc monotherapy in HIV-1-infected patients naive infected with R5- or DM/X4-using virus.

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

Fig. 3 Log_{10} X4 VL at baseline and end-treatment with maraviroc monotherapy in HIV-1-infected patients naive infected with R5- or DM/X4-using virus. X4-specific HIV-1 VL was calculated with the sequences with a frequency >1% and a FPR value below 3.75.

Fig. 4 Phylogenetic trees illustrating the phylogeny of 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 are shown in green and end-treatment in orange). **(A)** Four representative similar trees from 4 patients corresponding to R5 and DM/X4 tropism group. These phylogenetic trees had shown a distinct topology. Black triangles and gray circles show R5 and DM/X4 predicted tropism, respectively. **(B)** Four examples of 4 patients who showed phylogenetic trees with a combination of sequences from different treatment sampling time. If a sequence is maintained during treatment it is shown with two colors. Black triangles and gray circles show R5 and DM/X4 predicted tropism, respectively.