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HIV-1 Dynamics and Coreceptor Usage in Maraviroc-Treated Patients with Ongoing Replication

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There is evidence that HIV-1 evolution under maraviroc (MVC) pressure can lead to the selection of either X4-tropic variants and/or R5-tropic, MVC-resistant isolates. However, the viral dynamics of HIV-1 variants in patients with virological failure (VF) on MVC-containing regimens remain poorly studied. Here, we investigated the V3 loop evolution of HIV-1 on MVC in relation to coreceptor usage and the nature of HIV-1 quasispecies before MVC therapy using bulk population sequences and ultradeep sequencing. The majority of patients had no detectable minority X4 variant at baseline. The evolution of tropism was followed up until VF and showed three possibilities for viral evolution in these patients: emergence of preexisting X4 variants, *de novo* selection of R5 variants presenting V3 loop mutations, or replication of R5 variants without selection of known mutations.

Human immunodeficiency virus type 1 (HIV-1) infection involves an interaction between the viral envelope protein and the CD4 molecule. The V3 loop is then exposed and engages the coreceptor (CCR5 or CXCR4), which mediates membrane fusion. In the early stages of HIV-1 infection, CCR5 usage is predominant. The receptor switch to CXCR4 occurs often in infected individuals (around 40%) and is associated with faster disease progression (1). CCR5 inhibitors inhibit HIV-1 entry by blocking the CCR5 coreceptor. The binding of these small molecules to a cavity of the membrane domain of CCR5 stabilizes the coreceptor in a conformation which can no longer be recognized by the HIV-1 gp120 (2, 3). Maraviroc (MVC) is the first CCR5 inhibitor used for the treatment of CCR5-tropic HIV-1 infection (4). Two mechanisms of escape to MVC have been so far described *in vitro* and *in vivo* (5, 6). The first mechanism includes the selection of minority variants using the CXCR4 coreceptor; the second possibility is the emergence of CCR5-tropic resistant isolates which can still use the CCR5 coreceptor in the presence of the inhibitor. However, few studies have described the relative importance of the two mechanisms in MVC-treated patients (7, 8).

In this study, we described that the coreceptor switch occurred in only 30% of the patients, and we also characterized the genotypic evolution of HIV-1 isolates in patients with virological failure on MVC-based regimens.

MATERIALS AND METHODS

Study population. Included patients were screened for the maraviroc expanded-access protocol (MVC EAP) in France between January 2007 and August 2008 and received MVC associated with an optimized background therapy if the result of the phenotypic assay for coreceptor use determination was CCR5, using a previously validated assay (Trofile; Monogram Biosciences) (9). For some patients, a modified version with an optimized sensitivity of the assay was performed (ESTA) once it became available (10). Inclusion criteria for the MVC EAP were HIV-1 infection, age of ≥18 years, with previous antiretroviral therapy and virological failure with plasma HIV-1 RNA of >1,000 copies/ml. Patients from 18 centers in France were included in the virological GenoTropism study (11). Sociodemographic data, clinical data, and treatment histories were collected for all enrolled patients at the screening date. MVC-treated patients were followed up at baseline (M0) and at months 1, 3, and 6 on MVC-containing regimens (M1, M3, and M6). The patients had signed the MVC EAP informed consent form and were specifically informed about their participation in the GenoTropism study. The study was approved by the Comité Consultatif de Traitement de l'Information dans la Recherche Scientifique et Médicale and the Commission Nationale Informatique et Libertés. Only patients treated by MVC and with virological failure (VF; defined as plasma viral load [VL] above 50 copies/ml at month 3 or month 6) and available plasma samples at baseline and follow-up were studied here.

Virological methods. (i) Population sequencing. The sequence analysis comprising the complete V3 loop sequence was performed from plasma sampled at baseline MVC and at the time of follow-up with VL of >50 copies/ml. PCR primers and conditions and sequencing primers are described in the ANRS consensus techniques (http://www.hivfrenchresistance.org). PCR products were sequenced as described in the previous publication (11).

(ii) Ultradeep sequencing. *Env* C2V3 quasispecies were determined at M0 by 454 ultradeep sequencing (UDS; Roche). A 415-nucleotide fragment encompassing the V3 *env* region was generated by nested reverse transcription (RT)-PCR. The nested PCR was performed with the Expand high-fidelity Plus PCR system (Roche Diagnostics), with the following conditions: 1 cycle of 94°C for 2 min; 35 cycles of 94°C for 30 s, 60°C for 30 s, and 72°C for 3 min; followed by a final extension at 72°C for 7 min. The amplified PCR products were purified by using Agencourt Ampure PCR purification beads (Beckman Coulter, Brea, CA) and quantified with the Quant-iT Picogreen double-stranded DNA (dsDNA) assay kit (Invitrogen) on a LightCycler 480 (Roche). Pooled PCR products were clonally amplified on capture beads in water-in-oil emulsion microreactors, and pyrosequencing was performed by using PicoTiterPlate, following the standard approach for PCR amplicon sequencing. A total of 500,000 enriched DNA beads were deposited in the wells of a full GS Junior Titanium

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TABLE 1 Patient characteristics[a]

Patient no.	Time of follow-up	OBT	Viral load (log_{10} copies/ml)	$CD4^+$ cell count (cells/μl)	Tropism (population sequencing)	Tropism 454 (% X4)	Tropism TTT (pheno)
1	M0	DRV RAL ENF	5.8	20	R5	2.5	R5
	M1		5.5	12	X4	ND	R5X4
	M6		5.6	39	X4	ND	R5X4
2	M0	ETR RAL	5.2	336	R5	0	R5
	M3		2.8	376	R5	ND	ND
3	M0	3TC ETR DRV RAL	5.2	3	R5	0	R5
	M3		4.4	6	R5	ND	ND
	M6		4.6	8	R5	ND	ND
4	M0	TDF FTC RAL	5.4	130	R5	0	R5
	M6		2.1	342	R5	ND	ND
5	M0	AZT 3TC ABC ETR DRV RAL	5.2	15	R5	0.74	ND
	M1		5	70	X4	ND	X4
	M3		4.5	93	X4	ND	ND
	M6		3.2	193	X4	ND	ND
6	M0	DRV RAL	4.7	607	R5	0	R5
	M1		5	374	R5	ND	R5
	M3		3	460	R5	ND	R5
	M6		2.5	642	R5	ND	ND
7	M0	DRV RAL	5.2	607	R5	ND	ND
	M3		2.9	460	X4	ND	ND
8	M0	TDF FTC DRV	4.5	45	R5	0	R5
	M6		3	124	R5	ND	R5
9	M0	fAPV RAL ENF	4.5	131	R5	0	R5
	M3		3.3	132	X4	ND	X4
	M9		3.6	206	R5	ND	ND
10	M0	TDF FTC DRV	5.6	27	R5	1.3	R5
	M6		5.1	58	R5	ND	R5
11	M0	ABC 3TC DRV ENF	4.6	316	R5	0.3	R5
	M6		2.2	400	R5	ND	ND
12	M0	3TC ABC TDF ETR DRV ATV	5.2	82	R5	0	R5
	M3		3.5	383	R5	ND	R5
13	M0	3TC ETR DRV ATV RAL	2.4	276	R5	76.1	R5
	M3		2.3	457	R5	ND	R5
14	M0	DRV RAL	2.1	541	R5	ND	ND
	M3		2.4	390	R5	ND	ND
	M6		2.7	243	R5	ND	ND
15	M0	RAL	3	323	R5	0	R5
	M6		2.1	360	R5	ND	ND
16	M0	DRV	3.9	434	R5	0	R5
	M6		5.1	642	R5	ND	R5
17	M0	TDF FTC TPV RAL	5.5	131	X4	100	R5X4
	M1		4.3	121	X4	ND	ND
	M3		4.5	160	X4	ND	ND

[a] OBT, optimized background therapy; TTT, Toulouse tropism test; M0, follow-up at baseline; M1, M3, and M6, follow-up at months 1, 3, and 6 on MVC-containing regimens; DRV, darunavir; RAL, raltegravir; ENF, enfuvirtide; ETR, etravirine; 3TC, lamivudine; TDF, tenofovir; FTC, emtricitabine; AZT, zidovudine; ABC, abacavir; fAPV, fosamprenavir; ATV, atazanavir; R5, CCR5; X4, CXCR4; ND, no data.

PicoTiterPlate device and pyrosequenced in both forward and reverse directions. The 200 nucleotide cycles were performed in a 10-h sequencing run. For each sample, a fasta file containing nucleotide sequence data was obtained.

(iii) Phylogenetic analysis. Phylogenetic analyses were performed on V3 sequences, including both 454 sequences and population sequencing data, using the neighbor-joining method on Clustal W2 software and trees designed with the iTOL website (12).

(iv) Genotypic and phenotypic prediction of coreceptor usage. Coreceptor usage was predicted from V3 sequences using the Geno2pheno algorithm (http://coreceptor.bioinf.mpi-inf.mpg.de/) by selecting a false-positive rate (FPR) at 10% for sequences issued from population sequencing or a false-positive rate at 5.75% or 3.5% (13, 14) for the calculation of the X4 virus frequency for the 454 sequences.

Coreceptor usage was also determined at M0 and at follow-up by phenotypic analysis using a recombinant assay (Toulouse tropism test) (15).

RESULTS

Patients' characteristics. Seventeen patients were included in this study. Patient characteristics are detailed in Table 1. At baseline, before maraviroc-containing therapy, the median plasma HIV-1 RNA was 5.2 $\log_{10}$ copies/ml (range, 2.1 to 5.8), and at VF (M6), the median plasma HIV-1 RNA was 3 $\log_{10}$ copies/ml (range, 1.6 to 5.6). The median $CD4^+$ cell count was 131 cells/mm^3 (range, 3 to 607) at baseline MVC and 320 cells/mm^3 (range, 8 to 642) at M6. The antiretroviral drugs coadministered with MVC included nucleoside or nucleotide RT inhibitors for 9 patients, ritonavir-boosted protease inhibitors in 12 patients, a nonnucleoside RT inhibitor (etravirine) in 4 patients, an integrase inhibitor (raltegravir) in 11 patients, and a fusion inhibitor (enfuvirtide) in 3 patients.

Baseline MVC HIV-1 coreceptor usage. The inferred HIV-1 coreceptor usage from genotype was determined at baseline MVC from bulk HIV-1 plasma RNA. The genotypic analysis of the V3 loop, using the Geno2pheno (FPR, 10%) algorithm, showed an R5 tropism for all patients but one (Table 1). HIV-1 tropism using a phenotypic test (15) was determined at baseline MVC for 14 patients. These results were concordant with the genotypic determination, with R5 tropism for 13 patients and an R5X4 result for the patient with X4 genotypic tropism at baseline (and determined as R5 by Trofile assay).

The percentage of X4 variants could be determined by ultradeep sequencing of the V3 loop in 15 patients at baseline. The median number of analyzed sequences for each patient was 3,240 (range, 1,034 to 7,585). Out of 14 patients with R5 tropism from bulk population analysis, 9 had 0% of X4 isolates with UDS; 4 patients had minority X4 variants, ranging between 0.3% to 2.5% of the global population; and one patient had 76.1% of X4 variants. The patient with the X4 bulk population tropism was shown to have 100% of X4 variants by UDS.

Evolution of coreceptor usage at virological failure. Both population sequencing and phenotypic analysis showed a switch from R5 isolates at baseline to X4 isolates at VF in 4 patients, a stable R5 coreceptor use in 12 patients, and a stable X4 tropism in one patient. Of note, one patient (patient 9) with a switch from R5 isolate at baseline to X4 isolate at M3 reversed to R5 tropism at M6. Three (patients 1, 5, and 17) of 6 patients with minority X4 variants and 1/9 patient (patient 9) with 0% of X4 variants at baseline harbored X4 viruses at virological failure (Fisher's exact test, $P = 0.23$).

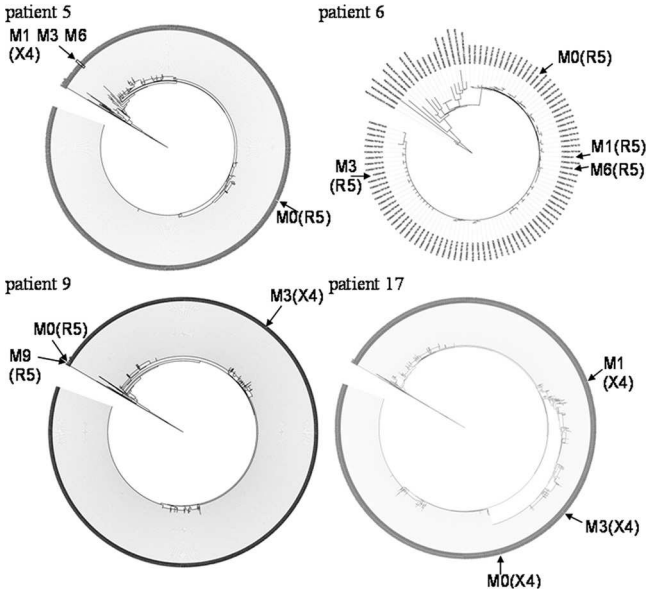


FIG 1 V3 quasispecies at baseline and at failure. Phylogenetic analysis, based on the neighbor-joining method, of the HIV-1 V3 region using 454 sequences at M0 and population sequences at times indicated for 4 patients. Corresponding tropisms are indicated between brackets. Only consensus sequences are represented for patient 6.

Evolution of V3 quasispecies on MVC. Phylogenetic trees could be constructed for 15 patients, with each tree including V3 loop sequences obtained from population sequencing at baseline MVC and at virological failure and sequences obtained by ultradeep sequencing at baseline MVC. This enabled us to define the most proximal UDS sequences to bulk sequences at baseline and at failure. Four examples of phylogenetic trees are shown in Fig. 1, showing different dynamics: a switch from the R5 isolate to a stable X4 population for patient 5, an R5 population for patient 6 with a low sequence variability, a selection of the X4 variant followed by a return to the baseline R5 population in patient 9, and an evolving X4 population in patient 17. In order to define specific mutations appearing at virological failure on MVC, we aligned the V3 loop amino acid sequences obtained at baseline and at failure and the proximal baseline UDS sequences for each patient (Fig. 2). For the three patients (1, 5, and 9) with selection of X4 variants, multiple mutations were selected at failure, including changes at critical amino acids 11 and 25. For patient 1 and 5, 2.5% and 0.74% of X4 variants were detected at baseline, including most proximal sequences to VF. For patient 9, no X4 minority variant was detected at baseline.

In a majority of patients, R5 sequences were found at virological failure. In 8 patients (patients 2, 4, 6, 8, 11, 12, 13, 16), a stable genotype was found over time. In three patients, the presence of multiple mutations was found in the V3 loop: mutations 13S, 22T, and 24A for patient 3; mutations 13P and 25Q for patient 10; and mutations 13S, 25G, 29D, and 34Y for patient 15. The corresponding mutations were not found in the most proximal UDS sequences at M0, suggesting that these mutations could have been selected later during MVC exposure.

DISCUSSION

In this study, we could characterize the evolution of HIV-1 quasispecies in patients enrolled in the GenoTropism study and having

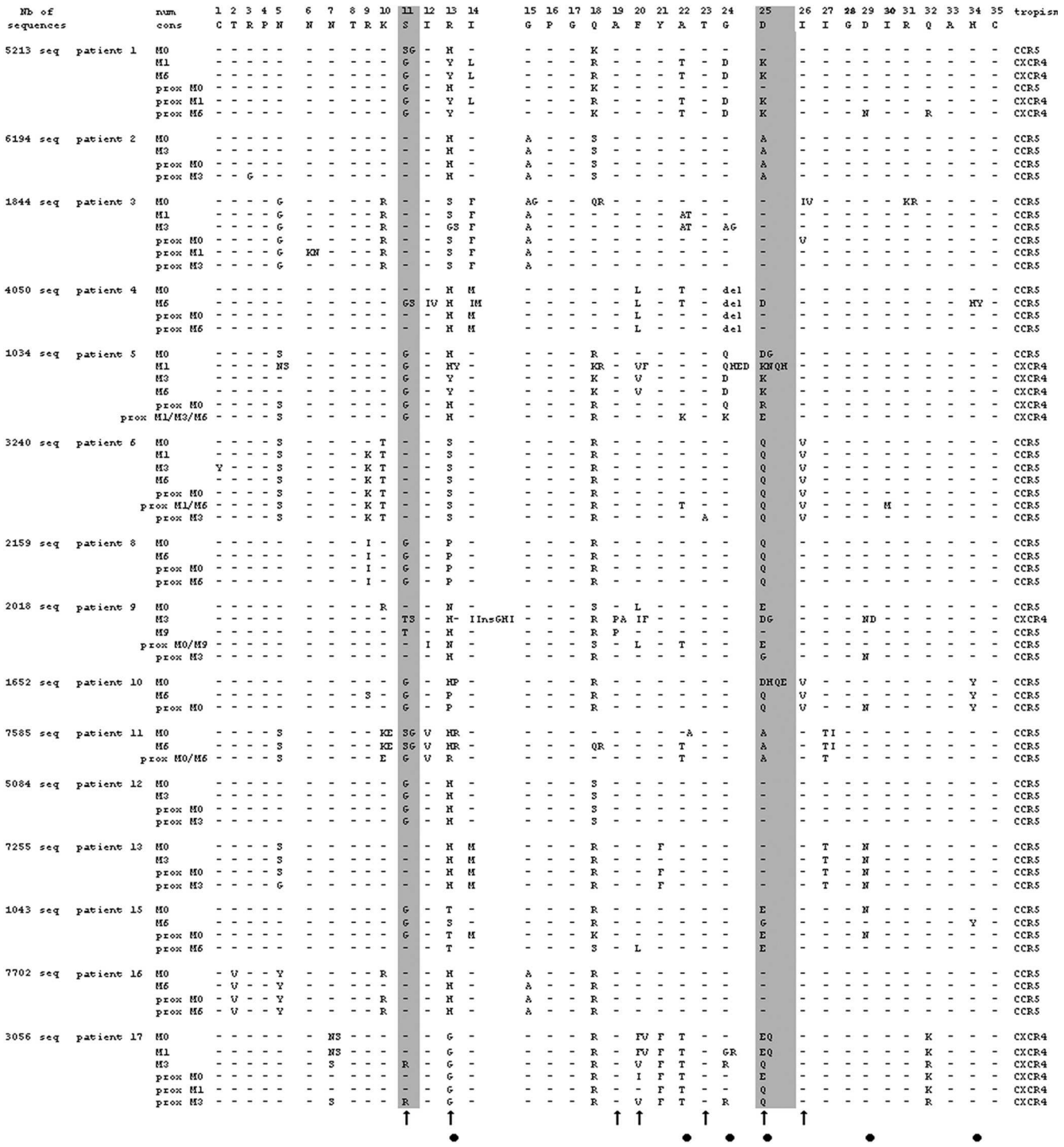


FIG 2 Virological evolution of the V3 region in the patients failing on MVC. The number of 454 sequences used for the analysis is determined in the first column. Prox M indicated the 454 sequence found to be the most closely related to the bulk population sequence at the studied time. For patient 10, no proximal sequence was identified to M6 bulk sequence. No 454 data were available for patients 7 and 14. Columns for amino acids at positions 11 and 25 are in gray. Black arrows indicate positions of known mutations. Black circles indicate positions of mutations observed in this study.

VF on MVC-containing regimens. The risk of VF has been shown to be independently associated with an X4 tropism at baseline MVC (determined from V3 sequences and the Geno2pheno algorithm), with a lower weighed genotypic sensitivity score (GSS), a higher baseline viral load, and a lower nadir of $CD4^+$ cells (11). Here, we focused on the evolution of coreceptor usage and the genetic evolution of V3 sequences in 17 patients with VF. Both genotypic and phenotypic tests showed that in all patients but one, the majority viral population used the CCR5 coreceptor at baseline. The diversity of *env* quasispecies at baseline MVC was studied

by UDS. In a majority of patients (9/14) with R5 tropism from bulk population analysis, no minority X4 variant could be found. However, 4 patients had minority X4 variants, and one was found to have majority X4 variants by UDS. The evolution of tropism could be followed up at VF, showing a switch from R5 isolates to X4 isolates in 4 patients, a stable R5 tropism in 12 patients, and a stable X4 tropism in one patient.

To investigate the dynamics of evolution of the *env* V3 loop between baseline MVC and VF, we constructed phylogenetic trees comprising all *env* baseline quasispecies and the bulk variants selected at VF. The switch from R5 to X4 tropism occurred in only 30% of the patients. In this case, multiple V3 mutations were selected at VF, including typical changes at amino acids 11 and 25 associated with X4 tropism. In 2/3 patients, preexisting X4 variants carrying all or most of these mutations were detected as minority variants at baseline, in agreement with a previous report showing that minority X4 variants could be selected by MVC (8). In patients with R5 tropism at VF, the picture was more complex. A majority of patients showed no evolution of V3 sequence between baseline MVC and VF. At least three mechanisms could be involved in these patients: (i) a nonoptimal adherence to antiretroviral therapy (ART) could have led to VF; however, most patients showed a decrease in plasma HIV-1 RNA between M0 and VF, which is not in favor of this possibility; (ii) a suboptimal efficacy of the whole ART regimen, despite partial efficacy due to MVC; (iii) resistance to MVC or CXCR4 usage could be encoded by genetic changes occurring out of the V3 loop, since changes in gp41 have been previously shown to influence coreceptor tropism (16, 17) and mutations in the gp120 V4 loop have been shown to modulate resistance to MVC (18). As an additional means of resistance, we could not exclude that MVC-resistant viruses utilized the drug-bound form of the receptors as previously described *in vitro* (5, 6, 19).

Three patients presented R5 isolates at VF with the selection of multiple mutations in the V3 loop. Because these mutations were absent from baseline sequences, they are likely to correspond to *de novo* viral evolution to use MVC-bound CCR5, as previously suggested (18). In the latter report, mutations P/T308H, T320H, and I322V in HXB2 gp120 (corresponding to amino acids 13, 23, and 25 in the V3 loop) were described in clinical isolates from patients with VF on MVC and were shown to code for resistance to MVC. In a previous report, mutations A316T, A319S, and I323V (V3 loop positions 19, 20, and 26), *in vitro* selected in the presence of MVC, were also shown to promote MVC resistance. Other patterns have been described in patients with VF in the MOTIVATE trial (20, 21) but without full molecular characterization. In our three patients, changes at position 13 were selected or present at baseline and VF, suggesting that this position may be critical for resistance to MVC. However, more data will be necessary to establish correlations between genotype and phenotype (and/or VF) for resistance to MVC, since mutation patterns seem to vary importantly among patients, suggesting a role of *env* variability background for establishment of resistance.

These V3 loop mutations should be further investigated to understand how they mediate MVC antiviral effects. We cannot also exclude a relationship between the background antiviral regimens and the likelihood of development of resistance against MVC. The numbers of patients were, however, too small to assess a significant relationship between the GSS and the different patterns observed at VF.

In conclusion, our study confirms two possibilities for viral evolution in patients with VF on MVC: emergence of preexisting X4 variants or *de novo* selection of R5 variants presenting V3 loop mutations. Besides these two patterns, the replication of R5 variants without selection of mutations warrants further pharmacokinetical and/or virological studies outside the V3 loop.

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