

# Research Letters

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## **Protease inhibitor monotherapy is not associated with increased viral replication in lymph nodes**

David Vinuesa<sup>a</sup>, Jorge Parra-Ruiz<sup>a</sup>, Natalia Chueca<sup>b</sup>, Marta Alvarez<sup>b</sup>, Leopoldo Muñoz-Medina<sup>a</sup>, Federico Garcia<sup>b</sup> and Jose Hernandez-Quero<sup>a</sup>

**There are concerns about residual viremia in sanctuary sites among patients on protease inhibitor monotherapy, so we aimed to study viro-immunological parameters in tonsil's lymphoid tissue of patients on highly active antiretroviral therapy (HAART) and on protease inhibitor monotherapy. Despite fully suppressed serum HIV viral load, we found viral replication in both groups; in addition, more patients had detectable proviral DNA among those on HAART, compared to those on protease inhibitor monotherapy ( $P=0.08$ ), supporting the absence of a deleterious effect of protease inhibitor monotherapy.**

Protease inhibitor monotherapy is being increasingly used with great efficacy and safety profile as a maintenance strategy [1–3]. This approach has some advantages like a reduction of nucleoside reverse transcriptase inhibitor (NRTI)-associated side effects [4], and cost-saving. In addition, protease inhibitor monotherapy may preserve, in almost all cases, future treatment options [5].

Despite these advantages, this strategy remains controversial, with many experts arguing against it because of concerns about poor central system penetration [6,7], higher risk of intermittent viremia [8], but without development of protease inhibitor resistance mutations [9,10], or an increased inflammatory state caused by HIV itself.

Lymphoid tissue constitutes the main reservoir of HIV. If concerns regarding a decreased activity of protease inhibitor monotherapy in viral sanctuaries were true, there had to be ongoing replication, so we aimed to investigate whether this increased viral replication exists in patients on fully suppressed protease inhibitor monotherapy.

All consecutive HIV-infected patients attending our Infectious Diseases Outpatient Clinic, older than 18 years old and with complete adherence to medication and outpatient visits were offered to participate. Patients on triple therapy (highly active antiretroviral therapy; HAART) or protease inhibitor monotherapy with undetectable serum HIV viral load during the past 12 months were eligible for the study.

The local Research Ethic Board of our Institution approved the study protocol and every patient signed an inform consent prior to any procedure.

After local anaesthesia, we obtained two biopsies from tonsils. One sample was sent to the Service of Microbiology, weighed and processed for nucleic acid extraction [QIAamp DNA mini kit (Quiagen, Iberia SL, Madrid, Spain)], HIV viral load quantification [Cobas Amplicor HIV-1 Monitor (Roche, Madrid, Spain)], and HIV-DNA testing [HIV DNA Cell test kit (ANRS, Biocentric, Bandol, France)]. The other biopsy was sent to Service of Pathology. Blood samples for immunological and virological markers were also obtained immediately before biopsies were obtained.

Thirty-one patients were included: 22 men (71%) and nine women (29%). Among them, 16 were on HAART (efavirenz/tenofovir disoproxil fumarate/emtricitabine) and 15 on protease inhibitor monotherapy [lopinavir/ritonavir (6) or darunavir/ritonavir (9)]. Baseline characteristics of patients are displayed in Table 1.

Seven patients (23%) had detectable HIV viral load in lymphoid tissue: four (25%) among patients on HAART and three (20%) among patients on protease inhibitor monotherapy [ $P=0.976$ , relative risk (RR) 1067, 95% confidence interval (CI) 0.73–1559]. We have data regarding tonsil-proviral DNA of 21 patients (68%). Tonsil-proviral DNA was detectable in 10 patients (48%), being more frequently detected among patients on HAART compared to those on protease inhibitor monotherapy (7/10, 70% vs. 3/11, 27%;  $P=0.08$ , RR 2567, 95% CI 0.901–7.31). In addition, median proviral DNA was higher among those on HAART compared to those on protease inhibitor monotherapy (392 vs. 0 copies/ml;  $P=0.057$ ).

In our study, we have shown ongoing viral replication in lymphoid tissue despite fully suppressed serum HIV replication. Despite a median of 4 years with HIV viral load below 20 copies/ml, up to 25% of our patients had detectable tonsil-HIV viral load, confirming persistent residual replication in viral sanctuaries. Ongoing viral replication did exist irrespective of antiretroviral therapy, being present in both groups, without any statistically significant difference.

Ongoing HIV viral replication in lymphoid tissue despite prolonged undetectable serum HIV viral load has already been reported by other authors [11,12]. However, to our knowledge, this is the first report to compare ongoing

Table 1. Basal characteristics of patients included.

	HAART (n = 16)	PI monotherapy (n = 15)	P
Age	44 (28)	41 (33)	0.29
Sex male (n, %)	12 (75)	10 (67%)	0.704
CDC stage (n, %)			0.594
A	9 (56)	8 (53)	
B	3 (19)	1 (7)	
C	4 (25)	6 (40)	
HCV (n, %)	1 (6.2)	3 (20)	0.333
CD4 <sup>+</sup> cell count	629 (844)	716	0.664
HIV viral load			0.482
<20 copies/ml	5	3	
<1 copies/ml	11	12	
CD4 <sup>+</sup> nadir	225 (350)	228 (443)	0.813
Route of transmission (n, %)			0.255
IVDUs	1 (6)	2 (13)	
MSM	10 (63)	8 (54)	
HTS	5 (31)	5 (33)	
Time on undetectability (years)	4 (4.2)	4 (4)	0.885

Data expressed in median (range), unless otherwise specified. CDC, Center for Disease Control; HAART, highly active antiretroviral therapy; HCV, hepatitis C virus; HTS, heterosexual; IVDUs, intravenous drug users; PI, protease inhibitor.

viral replication in lymphoid tissue of patients on HAART or on protease inhibitor monotherapy.

It is suspected that these viral sanctuaries serve as HIV reservoirs that can cause rebound of viremia after HAART discontinuation [13], so any attempt to find a cure for HIV infection has to achieve HIV eradication from these reservoirs. Lymph tissue is the main HIV reservoir, and residual viral replication is more frequently detected here than in other sanctuaries, such as genital secretions or cerebrospinal fluid [14]. Tonsils are an easily accessible lymphoid tissue, so we chose them to perform our study.

Proviral DNA HIV load (evaluated in resting memory CD4<sup>+</sup> T cells, monocytes and macrophages) has been related to the burden of residual disease as opposed to plasma RNA load that has been related to active infection [15]. In addition, residual plasma viremia has been correlated to the size of CD4<sup>+</sup> T-cell viral reservoirs [16]. In our study, we were able to test for tonsil's proviral DNA in 21 out of the 31 patients. Ten patients had detectable proviral DNA, and it was more frequently detected in patients on HAART than on those on protease inhibitor monotherapy, results that were very close to statistical significance. In addition, median values of proviral DNA load were higher in patients on HAART than in those on protease inhibitor monotherapy. Lambert-Niclot *et al.* [17] reported similar results, in a study from 160 patients included in the MONOI-ARNS 136 trial, for which blood cells were available. Despite an increased proportion of intermittent viremia among patients on protease inhibitor monotherapy (compared to that of patients on triple therapy), a similar variation of proviral DNA load was observed in both groups. In fact, the variation was lower in patients on boosted protease inhibitor monotherapy (0.35 log copies/10<sup>6</sup> leucocytes vs. 0.51 log copies/10<sup>6</sup> leucocytes for boosted protease inhibitor and triple therapy, respectively).

Proviral HIV DNA load has also been associated to specific antiretroviral therapy. Nicastri *et al.* [18] reported a decreased proviral HIV DNA among patients on a protease inhibitor-based HAART compared to patients on non-NRTI-based HAART. Although all patients were on HAART, it is noteworthy that they found similar results as we have reported, suggesting an enhanced activity of protease inhibitors against viral reservoirs. We cannot fully explain this apparently enhanced activity of protease inhibitor monotherapy, but a possible explanation could be the recently discovered multistep inhibition of protease inhibitors [19]. By exerting its activity at different levels in the viral life cycle, there could be a greater inhibition of HIV integration and persistence in viral reservoirs.

In conclusion, our study contributes to support the use of protease inhibitor monotherapy as a maintenance strategy as we did not find any differences in tonsil-viral replication, irrespective of the antiretroviral regimen (HAART or protease inhibitor monotherapy). We also detected proviral DNA more frequently in patients on HAART, compared to those on protease inhibitor monotherapy. Whether this may reflect a protective effect of protease inhibitor monotherapy merits further investigation with a larger sample size.

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### Conflicts of interest

There are no conflicts of interest.

<sup>a</sup>Service of Infectious Diseases, and <sup>b</sup>Service of Microbiology, Hospital Universitario San Cecilio, Granada, Spain.

Correspondence to Jorge Parra-Ruiz, MD, PhD, Servicio de Enfermedades Infecciosas, Hospital Universitario San Cecilio, Avda. Dr Olóriz 16, 18012 Granada, Spain.

Tel: +34 958 023 104; fax: +34 958 249 079; e-mail: jordi@ugr.es

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## High prevalence of subtype F in newly diagnosed HIV-1 persons in northwest Spain and evidence for impaired treatment response

Berta Pernas<sup>a</sup>, Marta Grandal<sup>a</sup>, Alvaro Mena<sup>a</sup>, Angeles Castro-Iglesias<sup>a</sup>, Angelina Cañizares<sup>b</sup>, David L. Wyles<sup>d</sup>, Soledad López-Calvo<sup>a</sup>, Sonia Pértiga<sup>c</sup>, Iria Rodríguez-Orsorio<sup>a</sup>, Jose Domingo Pedreira<sup>a</sup> and Eva Poveda<sup>a</sup>

**HIV-1 non-B subtype variants were found in 37.8% of 296 newly diagnosed persons in northwest Spain over the past 5 years. Subtype F was the most prevalent non-B subtype (29.6%) and displayed preferential transmission among MSM. Virologic response rates to antiretroviral therapy were lower among F subtypes compared to B subtypes at weeks 24 (31% vs. 78.3%), 48 (51.7% vs. 85.2%), and 96 (61.1% vs. 94.3%) of therapy. Subtype F was independently associated with virological response at 24 weeks.**

The prevalence of HIV-1 group M non-B subtypes has been increasing in Western Europe in recent years largely as a result of population movements [1]. Specifically, in native Spaniards, the rate of non-B variants increased from 1.5% in 2000–2002 to 11.4% in 2007–2010, with the circulating recombinant form CRF02\_AG (37%) being the most common non-B subtype in Spain [2]. Historically, the HIV epidemic in Galicia, a coastal region situated in northwest Spain, has been characterized by a high diversity of HIV genetic forms presumably due to international population movements through this area [3].

Natural polymorphisms at positions associated with resistance are frequently found among reverse transcriptase and protease sequences from HIV non-B variants [4]. Although the impact of these polymorphisms on treatment responses appears limited, recent studies have



demonstrated their relevance in some HIV variants with specific antiretroviral agents [5,6]. Since the prevalence of circulating HIV variants is a dynamic phenomenon and new antiretroviral agents are continually being introduced into the therapeutic arsenal against HIV infection, this issue requires continuous monitoring.

Herein, we describe the characteristics of all newly diagnosed HIV-1 persons in the past 5 years at our institution in northwest Spain serving 501 526 citizens. Patient demographics (age, sex, and risk behavior), and laboratory (HIV-RNA, HIV subtypes, HIV drug resistance and CD4<sup>+</sup> cell counts) and clinical parameters at the time of diagnosis were recorded. In addition, the response to antiretroviral therapy (ART) was retrospectively analyzed.

A total of 296 newly diagnosed HIV-1 patients were identified from 2009 to 2013 at our institution. HIV subtype could be determined in 230 patients. Non-B variants were found in 37.8% of patients with the following distribution: F (29.6%), C (2.6%), A (2.2%), CRF02\_AG (1.7%), G (0.9%), K (0.4%), and CRF01\_AE (0.4%). Subsequent analyses were performed comparing the two most prevalent subtypes seen in our population: subtype B (*n* = 143) and subtype F (*n* = 68).

Table 1 displays the main characteristics of this population at the time of diagnosis. Persons infected with subtype F were overwhelmingly male (97.1%) with a predominance of MSM compared to subtype B patients (81.8% vs. 45.8%, respectively; *P* < 0.001). Those infected with subtype F were also significantly younger than those infected with subtype B (36 vs. 39 years, respectively; *P* = 0.037). No differences were found with regard to nationality, with the majority in both groups being Spanish. Likewise, the percentages of subtype F versus B patients with an AIDS-defining illness (24.2% vs. 35%, respectively) or CD4<sup>+</sup> cell counts below 350 cells/ $\mu$ l (42.6% vs. 50.3%, respectively) were similar. The mean CD4<sup>+</sup> cell count was also comparable between the groups (384.04 vs. 378.03, respectively); however, HIV-RNA levels at the time of diagnosis were higher among subtype F patients compared to subtype B patients (5.3 vs. 4.9 log copies/ml, respectively; *P* = 0.002). The rate of transmitted drug resistance during the study period was 4.2%, and resistance was only found in subtype B sequences.

After diagnosis, 80.8% of subtype F and 72.7% of subtype B patients initiated ART. The rates of virological response after ART initiation were retrospectively evaluated comparing both groups of patients (Table 1). The mean time to initiation of ART was significantly shorter among patients infected with subtype F variants (10.53 vs. 17.60 months, respectively; *P* < 0.001). No differences were observed with regard to the composition of the initial ART regimen between subtype F and B patients: two nucleoside reverse transcriptase inhibitors (NRTI) +

one non-nucleoside reverse transcriptase inhibitor (NNRTI) (55.4% vs. 47.5%), two NRTI + one protease inhibitor (35.7% vs. 46.5%) and two NRTI + one integrase inhibitor (INI) (8.9% vs. 6.1%).

Virologic response, defined as achievement of HIV-RNA below 50 copies/ml, was assessed at 24, 48, and 96 weeks after ART initiation. Interestingly, the rates of virologic response were significantly lower among patients infected with subtype F variants compared to subtype B variants at weeks 24 (31% vs. 78.3%), 48 (51.7% vs. 85.2%), and 96 (61.1% vs. 94.3%). Importantly, the virologic response did not vary based on the composition of the ART regimen (e.g. protease inhibitor vs. NNRTI). However, similar mean increases in CD4<sup>+</sup> cell counts 48 and 96 weeks after ART initiation were seen in both groups (Table 1).

In multivariate analysis, infection with a subtype F variant [odds ratio (OR) 14.8 (2.5–16.7), *P* < 0.001] and a high baseline plasma HIV-RNA level [OR 11.4 (1.7–7.9), *P* = 0.001] were independent predictors of a poor virologic response at 24 weeks after adjusting for ART initiation delay and baseline CD4<sup>+</sup> cell count.

Subtype F accounts for less than 1% of HIV-1 infections worldwide, primarily being found in Africa (Congo) and South America (Brazil). In Europe, subtype F has an unusually high prevalence in Rumania (>70%) due to parenteral transmission in children during the late 1980s [7]. Recently, subtype F HIV-1 infection has also been recognized among Italian heterosexual men [8]. A rapid expansion of HIV-1 subtype F among MSM in Galicia was reported in newly diagnosed patients during 2010–2011 [9]. Herein we confirm the continued spread of subtype F among MSM in northwest Spain and, for the first time, provide evidence of a suboptimal response to ART when compared with subtype B patients.

In regard to treatment response, some studies have highlighted the relevance of natural polymorphisms conferring resistance to specific antiretrovirals among ART-naïve individuals infected with non-B subtypes [5,6]. In this study, no significant differences were observed in the prevalence of major polymorphisms associated with resistance to antiretrovirals within reverse transcriptase or protease sequences from subtype F and B viruses. As expected, some differences in minor polymorphisms were found. The variant V106I, which is associated with low-level resistance to the NNRTI etravirine [10,11], was found more frequently among subtype F (compared to subtype B) sequences (83.8% vs. 3.5%, respectively; *P* < 0.001). Similarly, variants L10V, M36I, and L89M at positions associated with resistance to protease inhibitors were more frequent among subtype F (compared to subtype B) sequences (91.2% vs. 6.3%; 89.1% vs. 32.2%; and 91.2% and 8.4%, respectively; *P* < 0.001). The presence of these polymorphisms has been associated with a reduced susceptibility to the

**Table 1. Characteristics of newly HIV diagnosed patients at the time of diagnosis and response to antiretroviral therapy (subtypes B versus F).**

Characteristics	N = 211		P
	Subtype F (n = 68)	Subtype B (n = 143)	
Male (%)	97.1	86	0.014
Age	36.23 ± 9.9	39.28 ± 9.7	0.037
Routes of HIV transmission (%)			<0.001
Heterosexual	16.7	47.3	
Homosexual	81.8	45.8	
Others (including IDU)	1.5	6.9	
Spanish (%)	76.5	83.2	0.263
AIDS-defining diseases (%)	24.2	35	0.121
Late diagnosis (%)	42.6	50.3	0.295
Mean CD4 <sup>+</sup> at diagnosis time	384.04 ± 231.201	378.03 ± 316.800	0.512
Mean HIV-RNA (log copies/ml) at diagnosis time	5.3 ± 0.85	4.96 ± 0.81	0.002
Transmitted drug resistance (%)	0	4.2	0.180
Response to ART	n = 55	n = 104	
ART initiation delay after diagnosis (months)	10.53 ± 16.729	17.60 ± 22.485	<0.001
Regimens at ART initiation (%)			0.401
2 NRTI + 1 NNRTI	55.4	47.5	
2 NRTI + PI	35.7	46.5	
2 NRTI + INI	8.9	6.1	
Patients with HIV-RNA <50 copies/ml after ART initiation			
At 24 weeks on therapy (%)	31	78.3	<0.001
At 48 weeks on therapy (%)	51.7	85.2	<0.001
At 96 weeks on therapy (%)	61.1	94.3	0.005
Mean CD4 <sup>+</sup> cells/μl increase after 48 weeks after ART	271.34 ± 186.333	260.71 ± 190.891	0.788
Mean CD4 <sup>+</sup> cells/μl increase after 96 weeks after ART	256.35 ± 190.545	315.58 ± 275.593	0.432

ART, antiretroviral therapy; IDU, injecting drug user; INI, integrase inhibitor; NNRTI, non-nucleoside reverse transcriptase inhibitor; NRTI, nucleoside reverse transcriptase inhibitor; PI, protease inhibitor.

protease inhibitor tipranavir in subtype F specimens (fold-changes <2.7); however, no impact on darunavir susceptibility was found [5]. Since none of the patients in this study were treated with etravirine or tipranavir-based regimens, the presence of polymorphisms associated with reduced susceptibility to these drugs does not account for the poor virologic response observed among subtype F patients. Therefore, the susceptibility of subtype F variants to antiretroviral agents currently used for the treatment of HIV infection needs to be assessed.

Of note, in the study published by Poveda *et al.* [5], subtype F possessed the highest replication capacity (performed by Phenosense assay; Monogram Biosciences, San Francisco, California, USA) among the HIV subtypes tested. This finding is consistent with the significantly higher baseline plasma HIV-RNA levels observed among subtype F patients in our population. However, in the multivariate analysis accounting for baseline viral load, subtype F infection retained a significant association with delayed virologic response.

Poor adherence to ART in the group of subtype F patients is another possible explanation for the lower rates of virologic response. Although a direct measurement of adherence is not available in this study, several studies have reported a higher degree of adherence to ART and HIV care among the MSM population than in other HIV-risk groups [12]. Moreover, similar increases in CD4<sup>+</sup> cell counts were seen after ART initiation in both groups; therefore, poor adherence among subtype F patients is an

unlikely explanation for the poor virologic outcomes. Finally, there was no difference between the groups in terms of the number of different ART regimens initiated during the study period.

In summary, subtype F is the most prevalent non-B subtype among newly diagnosed HIV-1-infected persons in northwest Spain, with preferential transmission among MSM. For the first time, we have identified a significantly slower virologic response to ART among subtype F patients. Subtype F and a high baseline HIV-RNA were the major determinants of a poor virologic response to ART. Factors not associated with virologic response include: the timing of ART initiation, baseline CD4<sup>+</sup> cell count, and the initial ART regimen (NNRTI vs. protease inhibitor). Given the potential clinical implications of these results, additional studies are warranted to identify the reasons for poor ART virologic response in subtype F patients.

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### Conflicts of interest

There are no conflicts of interest.

<sup>a</sup>Grupo de Virología Clínica, <sup>b</sup>Servicio de Microbiología, <sup>c</sup>Unidad de Epidemiología Clínica y Bioestadística, INIBIC-Complejo Hospitalario Universitario de A Coruña, A Coruña, Spain, and <sup>d</sup>Division of Infectious Diseases, University of California, San Diego, La Jolla, California, USA.

Correspondence to Dr Eva Poveda, Grupo de Virología Clínica, Instituto de Investigación Biomédica de A Coruña (INIBIC), Complejo Hospitalario Universitario de A Coruña (CHUAC), As Xubias s/n, A Coruña 15006, Spain.

Tel: +34 981 17 63 99; fax: +34 981 17 63 98;

e-mail: eva.poveda.lopez@sergas.es

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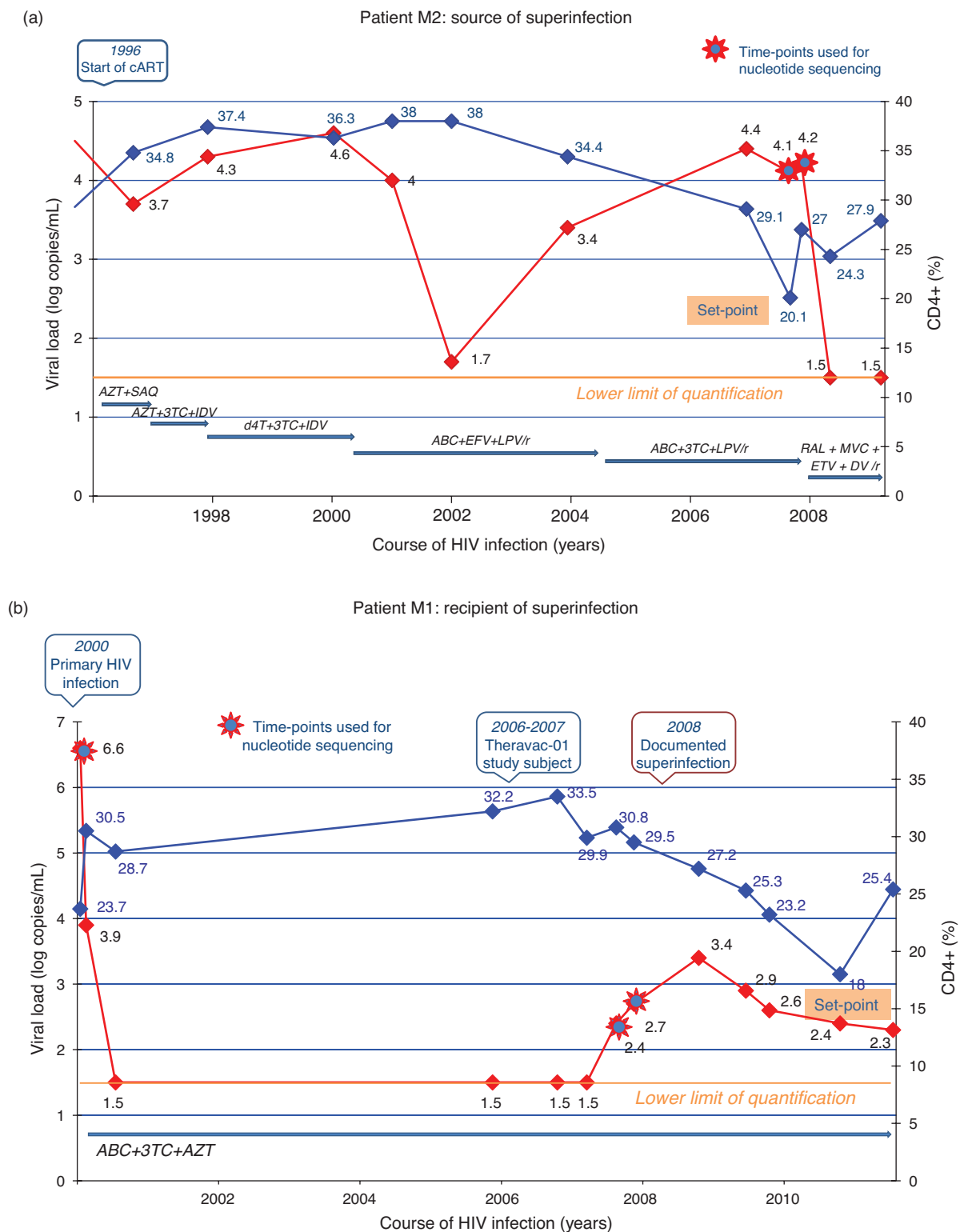
### HIV-1 superinfection with a triple-class drug-resistant strain in a patient successfully controlled with antiretroviral treatment

Erika Castro<sup>a,c</sup>, Hong Zhao<sup>b</sup>, Matthias Cavassini<sup>a</sup>, James I. Mullins<sup>b</sup>, Giuseppe Pantaleo<sup>c</sup> and Pierre-Alexandre Bart<sup>c,d</sup>

**We report a case of HIV-1 superinfection (HSI) with a clade B, triple-class resistant virus in a patient successfully controlling viremia with continuous combination antiretroviral therapy started 8 years earlier during primary HIV infection. The course of HIV infection prior to HSI was monitored in both the source partner and recipient (8 and 11 years, respectively) and 4 years following HSI. This case report demonstrates re-infection with HIV-1 despite effective combination antiretroviral therapy.**

HIV-1 superinfection (HSI), that is, infection with a second strain after the first has been established, has been reported since 2002, mainly involving HIV group M clades [1–6], and in a smaller proportion as inter-group recombinant forms [7,8]. Overall, HSI has been most often observed in untreated patients, during treatment interruption, and in seroconcordant couples with poor viral suppression [9]. Here, we report the onset and 4-year follow-up of HSI with a triple-class resistant clade B virus in a man on effective combination antiretroviral therapy (cART).

Briefly, blood samples were obtained from two men (M1 and M2) chronically infected with HIV-1 and sexual partners since 2006. M2 was diagnosed with HIV infection in 1994 at stage A2 with full Western blot seroconversion, and developed triple-class antiretroviral drug resistance as a consequence of weak adherence to ART, leading to virologic failure. M2 experienced uncontrolled viremia (range 3–4 logs of viral RNA) until September 2008 when a salvage treatment regimen reduced his viral load to undetectable limit for the first time (Fig. 1a). M1 was diagnosed in 2000 with primary HIV infection, initiated cART in 2000, and remained on cART with undetectable viremia and no drug resistance mutations through the end of 2007 (Fig. 1b). cART first



**Fig. 1.** (a) and (b) Patients' viral load and CD4<sup>+</sup> cell count kinetics during follow-up period in association to cART regimes. (c) Maximum likelihood trees of HIV-1 M1 and M2 *env* and *gag* sequences. 1.6 kb *env* and 1.5 kb *gag* sequences were aligned using the MUSCLE option in Seaview ([10]) and PhyML trees generated using the DIVEIN [11]. Tip labels are colour-coded (see Key) and indicate the sample and time point (mmyy) from which plasma samples were derived for sequencing. The scale bar indicates a genetic distance of 0.02 (2% distance). The branches extending to the M107/00 clusters in both trees were truncated for clarity of presentation, with the *gag* branch corresponding to a distance of 0.108 (10.8%) and the *env* branch corresponding to 0.400 (40%). cART, combination antiretroviral therapy.



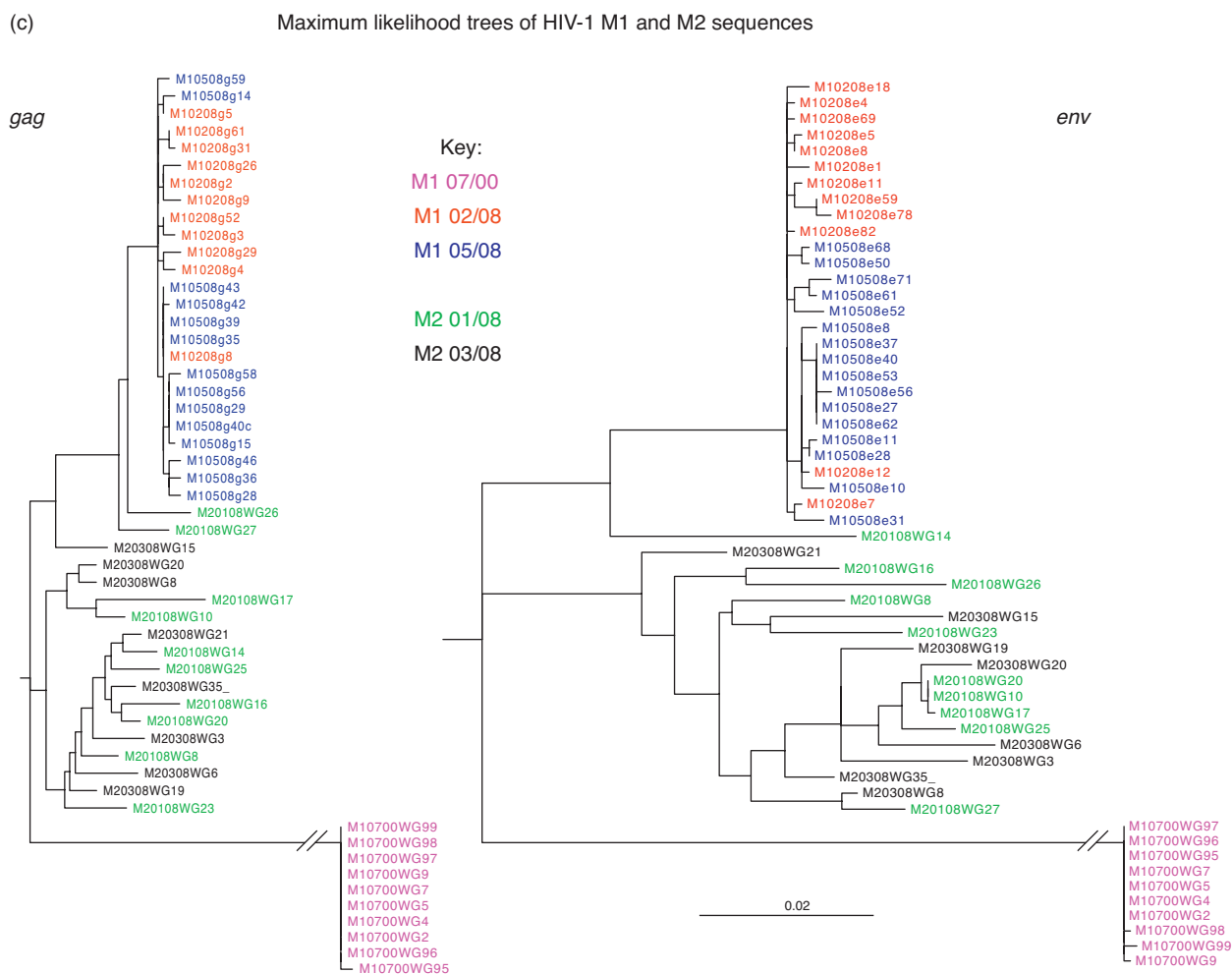


Fig. 1. (Continued).

regimen consisted of zidovudine (ZDV) + lamivudine (3TC) + efavirenz (EFV). In 2001, EFV was replaced by boosted lopinavir (LPV/r) due to EFV neurologic intolerance. The therapy was simplified in 2002 with the association of ZDV/3TC/abacavir (ABC) (Trizivir). M1 was also a vaccine-recipient in the therapeutic HIV vaccine trial TheraVac01 [12]. Briefly, the trial was an open-label one-arm study that took place in Lausanne, Switzerland. All patients ( $n=10$ ) were immunized with New York vaccinia virus expressing HIV-1 clade B antigens (NYVAC-B) (Gag/Pol/Nef polygene of HIV IIIB, and Env clade B of HIV BX08) intramuscularly (10E7.4 cell culture infectious dose 50%/ml) at weeks (W)0 and W4. M1 was assigned the code TH#04 and was immunized on May 17 (W0) and on June 14 (W4) 2006, respectively.

In February 2008, M1 presented with a plasma viral load of 280 copies/ml, which increased over the following year. Genotypic analysis from 2008 onward revealed 25 new drug-resistance mutations to nucleoside reverse transcriptase inhibitor (NRTI) (6), non NRTI (NNRTI) (5) and

protease inhibitor (14). Of note, this resistance profile shared 22 of 23 mutations (96%) found contemporaneously in M2. The shared mutations included NRTI resistance mutations 41L, 74I, 184V, and 215Y; NNRTI mutations 98G, 103N, and 108I; and protease inhibitor mutations 10V, 13V, 20R, 32I, 33I, 46I, 47V, 50V, 71I, and 77I. M1 also developed 75T (NRTI), 115/Y (NNRTI) and 82A (protease inhibitor) polymorphisms.

We obtained viral gene sequences from M1 from July 2000 [ $n=1$ , 1 ~9 kb near full-length genomes (NFLG)], February 2008 ( $n=12$  gag and env genes) and May of 2008 ( $n=14$  gag and  $n=16$  env genes), and from M2 in January ( $n=10$  NFLG) and March of 2008 ( $n=7$  NFLG). All 86 viral sequences were assigned to clade B. Additionally, all sequences from M1 obtained from 2008 clustered with M2 sequences from the same year and were phylogenetically unrelated to M1 sequences from 2000 (data not shown). This indicated HSI of M1 with substantial or complete replacement with virus from M2. No recombination between the M1 and M2 strains was observed.



The viral load of patient M1 increased from undetectable to above 3 logs after HSI, decreasing progressively from 2009 to 2012 without cART modification. A continuous drop of CD4<sup>+</sup> cells of at least 10% was observed from time of HSI detection in February 2008 (30.8%) through April 2011 (18%). However, in April 2012, the CD4<sup>+</sup> cell count for M1 recovered to 25.4% (757 cell/ $\mu$ l) (Fig. 1b). The follow-up for patient M2 was taken over by a general practitioner in 2009 after three consecutive undetectable viral loads following start of salvage antiretroviral treatment (Fig. 1a).

We detected superinfection and replacement by HIV-1 clade B triple-class resistant virus in a patient on long-term cART controlled infection, with the initial indicator of HSI being a detectable and increasing viral load.

Other resistance mutations not found in the superinfecting strain also emerged following HSI, though their origin is unclear. They could have been present as minority populations prior to superinfection or transmitted with the superinfecting strain but below the detection level of our assessment of M2's quasiespecies (no additional specimen was available for massively parallel sequencing). Despite HSI with a triple-class resistant virus, the ART for M1 remained unchanged. During the 4 years of additional follow-up, a continuous drop of viral load occurred, followed by CD4<sup>+</sup> recovery in the past year (Fig. 1b). The impact of HSI in CD4<sup>+</sup> decline and disease progression following subtype B coinfection was initially suggested by Gottlieb *et al.* [13]. Evidence of CD4<sup>+</sup> T-cell decline as the initial indicator of HSI was also reported in untreated patients during primary HIV infection as well as in untreated elite controllers [14–16].

In contrast to previous studies of superinfection among long-term known seroconcordant couples undergoing ART [9], this study underscores the fragile chemoprophylactic barrier exerted by ART despite excellent adherence. Apart from the complex resistance profile of the superinfecting strain present in patient M2, we cannot rule out the role of viral escape to pre-existing cytotoxic T-lymphocyte or antibody responses in the establishment of the second infection, which involves the same clade, and therefore, to some extent challenged similar immune signatures in the superinfected individual, as reported elsewhere [17,18]. Of interest, the HIV-specific T-cell responses of M1 were enriched following NYVAC-B immunizations (W0 and W4) and 21 months prior to superinfection [12]. Briefly, two novel Env/Pol vaccine-induced responses emerged at W2 and remained present throughout the study with a clear decline by W48 [12], greater than 1 year prior to HSI. The protective role of these vaccine-induced responses to prevent a second infection with a resistant virus, as well as their longevity, remains unproven. Haplotypes such as human leukocyte antigen (HLA)-B3503 have been shown to be associated with HIV-1 superinfection susceptibility as a

consequence of late or weak immune response priming [16]. In this regard, patient M1 carries HLA haplotype A\*30/23, B\*35/44, DRB1\*14/04 (performed by PCR-SSO using LabType kit on the Luminex System). He also had no CCR5-delta 32 mutations (in-house PCR modified from Wilkinson *et al.* [19]).

In summary, patient M1's superinfection onset agrees with the current knowledge on host factors and re-exposure to a resistant strain, and demonstrates the previously unexpected scenario of re-infection during well established chronic infection, despite continuously suppressed viremia with cART started during primary HIV infection.

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Sequence data: Nucleotide sequences were deposited in GenBank and are available under accession numbers KC797171–KC797229.

## Conflicts of interest

There are no conflicts of interest.

<sup>a</sup>Service of Infectious Diseases, Centre Hospitalier Universitaire Vaudois, Lausanne, Switzerland, <sup>b</sup>Departments of Microbiology, Medicine and Laboratory Medicine, University of Washington School of Medicine, Seattle, Washington, USA, <sup>c</sup>Laboratory of AIDS Immunopathogenesis, Service of Immunology and Allergy, and <sup>d</sup>Service of Internal Medicine, Centre Hospitalier Universitaire Vaudois, Lausanne, Switzerland.

Correspondence to Erika Castro, Addiction Medicine Unit Saint Martin, Service of Community Psychiatry, Centre Hospitalier Universitaire Vaudois, University of Lausanne, Rue Saint-Martin 7, 1003 Lausanne, Switzerland.  
E-mail: erika.castro-bataenjer@chuv.ch

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