

Clinical, Virologic and Immunological Outcomes in a Cohort of Long-Term Non-Progressor HIV Infected Patients, Southern Brazil

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Abstract

Objectives: We evaluated the HIV-1 subtype diversity, clinical, genetic and epidemiological profiles of a cohort of long-term non-progressor (LTNPs) followed-up at a referral hospital in southern Brazil.

Methods: This prospective study included patients who had more than eight years of HIV-1 diagnosis without antiretroviral therapy (ART). Clinical and epidemiological profiles of LTNPs were obtained from interviews and medical records. Periodic blood draws were taken to determine HIV-1 genetic variability and host genetic patterns.

Results: The study included 22 LTNPs, corresponding to 1.57% of patients followed-up at the Infectious Diseases Division. The gender distribution was nearly homogeneous, median age was 45 years; 18% were elite controllers, 23% were viremic controllers and 59% were non-viremic controllers. Three out of 22 patients were heterozygous for the CCR5Δ32 genotype. In most study patients, receptor use was consistent with an R5 phenotype. HIV-1 genotyping showed subtype C in 50% (11/22) of patients, subtype B in 32% (7/22), and the recombinant forms BF and BC in 14% (3/22) and 4% (1/22), respectively. There was a significant association of subtype C with female patients, and LTNPs patients infected with subtype C had lower viral loads compared with those infected with subtype B.

Conclusions: The HIV/AIDS epidemic in Brazil is complex, and there are variations in the subtype distribution. This is the first study of LTNPs in the southern region of Brazil, and the data obtained will help to characterize this group and aid in determining the probable mechanisms associated with delayed clinical progression.

Keywords: HIV; Long-term non-progressor; Elite controllers

Introduction

Among HIV+ individuals, there is a group with a markedly slower progression to AIDS; these individuals, called long-term non-progressor (LTNPs), present an asymptomatic stage of the disease for several years without antiretroviral therapy (ART) use, with high CD4+ T cell counts and low or undetectable viral loads. The control of viral replication and limited progression observed in LTNPs has been shown to be associated with various host, immune, and viral factors. Regarding the associated host characteristics, some HLA alleles, particularly HLA B*27 and B*57, have been found to be overexpressed in LTNPs. Other genetic markers, such as the heterozygous Δ32CCR5 genotype and CCR5 downregulation, lead to higher levels of chemokines, which activate receptor internalization, have been shown to be related to a longer clinical evolution and viral replication control. From a virological point of view, significant deletions and deleterious mutations have been found in viruses isolated from LTNPs [1-4].

Studies suggest that clinical damage in HIV-1 infected patients can vary depending on differences in viral and host characteristics [3,5]. HIV-1 subtypes may exhibit differences in transmission rates, disease progression, neurotoxicity, antiretroviral treatment failure profiles, and the accuracy of viral load measurements [6-8]. The HIV/AIDS epidemic in Brazil is complex, subtype B is the most common, followed by subtypes C and F1 and the recombinant forms BC and BF1. However, divergent distribution patterns of HIV-1 subtypes have been reported in distinct regions of the Country [9,10]. In the northern region, subtype B is the most common, followed by subtype F. In the northeast also predominant subtype B followed by F subtype and recombinant forms, which is the same as that reported in the midwest region of the country [11].

In the southeast, where the HIV epidemic began, there is a higher

frequency of genotype B, followed by F and the recombinant forms, and a lower percentage of subtype C [12]. In the southern region, there is a very different profile distribution, with a progressive increase of subtype C infection (20–50% of cases), especially in women and newly infected patients [10,13-16]. The aim of the present study was to evaluate the HIV-1 subtype diversity, and the clinical, genetic, and epidemiological profiles of a cohort of LTNPs followed-up at a referral hospital in Curitiba, Paraná, southern Brazil.

Material and Methods

Study subjects

This is a prospective cohort study carried out in the Infectious Diseases Division at a tertiary care academic center Hospital de Clínicas/Universidade Federal do Paraná (HC/UFPR) in Curitiba, southern Brazil. The study included patients who had more than eight years of HIV-1 diagnosis without ART. The clinical and epidemiological profiles of the LTNPs were obtained from interviews and medical

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record reviews. Periodic blood samples were obtained by standard venipuncture in ethylenediamine-tetra-acetic acid (EDTA) tubes, and routine immunological and molecular assays to assess HIV infection were performed. The Institutional Review Board approved this study (IRB: #003004912.7.0000.0096), and all included patients provided written informed consent.

PCR amplification, sequencing, and HIV subtyping

DNA was extracted from 10^7 peripheral blood mononuclear cells (PBMCs) separated on a Histopaque® 1077 (Sigma-Aldrich) gradient using a commercial extraction kit, according to the manufacturer's protocol. Proviral DNA amplification and sequencing of the *pol* (nucleotides 2077–3574 in HXB2) and *env* (nucleotides 7001–7667 in HXB2) genes were performed as previously described [17,18]. The PCR products generated were sequenced using the BigDye® Terminator v3.1 Cycle Sequencing Kit (Applied Biosystems, Foster City, CA, USA), according to the manufacturer's instructions, in an Applied Biosystems® 3130 Genetic Analyzer. Nucleotide sequences were edited using the DNASTAR Lasergene SeqMan program (version 7.0; Dnastar, Inc., Madison, WI). The subtype assignment of sequences was confirmed using the REGA HIV subtyping tool v.3.0 (<http://hivdb.stanford.edu/RegaSubtyping/stanford-hiv/typingtool/>), and by performing maximum likelihood (ML) phylogenetic analyses with HIV-1 group M subtype reference sequences of HIV-1 subtypes available in the *Los Alamos* database, (<http://www.hiv.lanl.gov/components/sequence/HIV/search/search.html>), constructed under the best-fit model using the algorithm implemented in MEGA 6.0 package [19]. Recombinant profiles were inferred by bootscanning analysis using SimPlot 3.5.1 software [20].

Characterization of CCR5Δ-32 genotype and phenotype

Analysis of the CCR5 polymorphisms in PBMC DNA were

analyzed by PCR as previously described [21]. The primers amplified a 225-nt gene fragment for the wild-type allele and a 193-nt fragment for the CCR5Δ-32 allele, and these fragments were separated on a 2% TBE agarose gel.

Co-receptor usage predictions based on V3 sequences were performed by the 11/25 rules using Geno2pheno (<http://coreceptor.bioinf.mpihb.mpg.de/cgi-bin/coreceptor.pl>) with a false-positive rate of 0.1 and the Phenoseq platform (<http://burned.edu.au/phenoseq>).

Statistical analyses

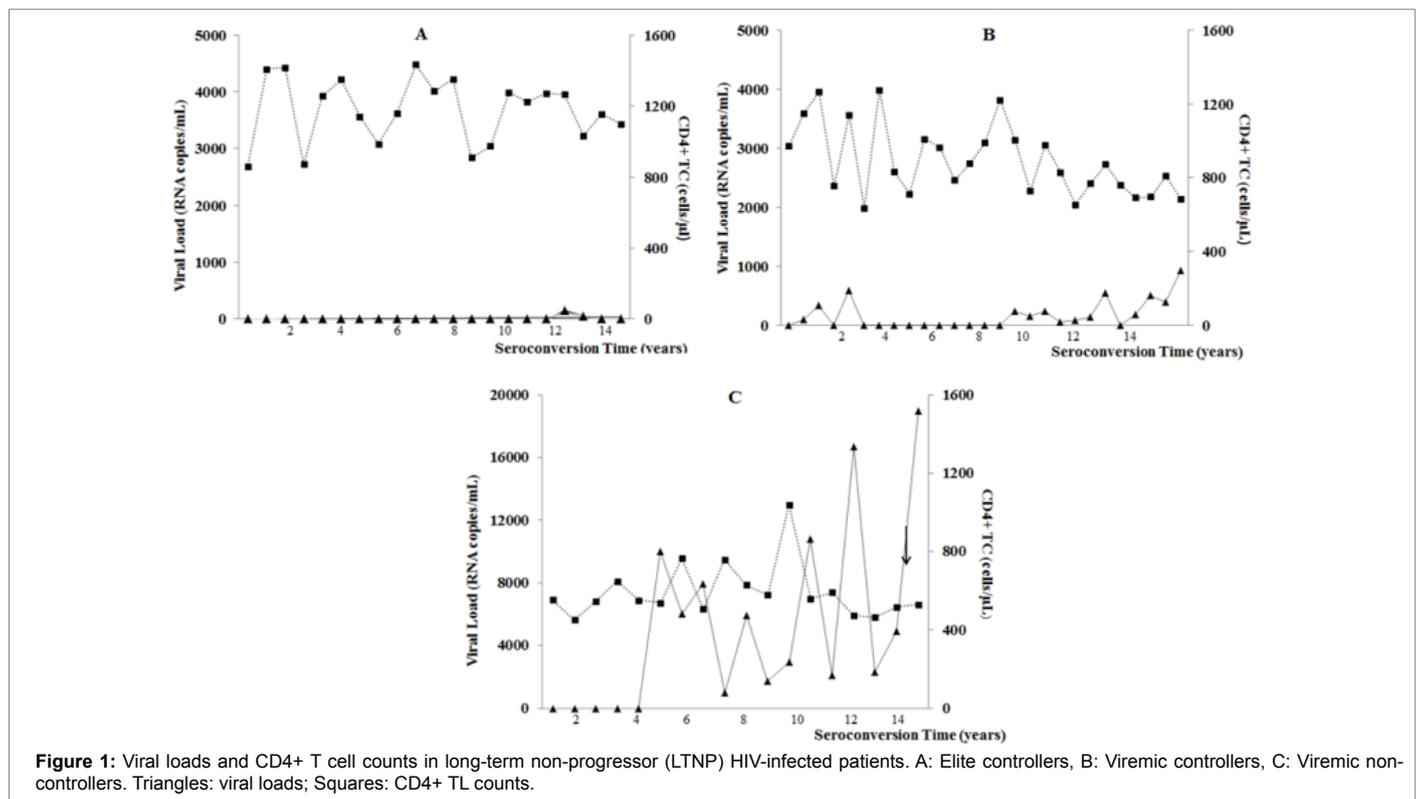
HIV-1 subtype, demographic, epidemiological, clinical, and genetic data were compiled and analyzed using GraphPad Prism® software version 5.03 (Graph Pad Software Inc., La Jolla, CA, USA). Fisher's exact test or the χ^2 test was used to assess between-group differences, and the Mann-Whitney test was used for continuous variables. Results for continuous data are expressed as median \pm interquartile range. All *p*-values are two-tailed, and a *p* value of less than 0.05 was considered significant.

Nucleotide sequence accession numbers

The sequences reported in this study were deposited in GenBank under accession numbers KT203233 to KT 203271 and KT260070 to KT260117.

Results

To date, 22 patients, corresponding to 1.57% of the total patients followed-up at the Infectious Diseases Division of HC/UFPR were identified as LTNPs. The gender distribution was almost homogeneous (54% female), and the median age at the time of the study was 45 years (range, 12–62y). The risk behavior for HIV-1 infection was sexual activity in 14 (63%; 71% were heterosexual), vertical infection in 5



(23%), and injectable drug use in 3 (14%) cases. The median time of HIV-1 diagnosis was 13.5 years (IQR 12;16), and the median CD4+ T lymphocyte count was 684 cells/mm³ (IQR 569;890).

Classification of long-term non-progressor (LTNPs)

According to the summarized classification of Casado and collaborators [4], 4/22 (18%) patients were classified as elite controllers (EC), with undetectable viral loads in nearly all tests, 5/22 (23%) individuals presented low viral loads (up to 2000 copies/mL) and were classified as viremic controllers, and the remaining LTNPs (13/22; 59%) presented variable viral loads in evaluated scores (Figure 1). Plasma viral load was systematically determined from

different detection models proposed by the network of public health laboratories of the Brazilian Ministry of Health (Cobas® Amplicor HIV-1 Monitor assay-Roche Diagnostics, and VERSANT® HIV-1 RNA 3.0 assay- Siemens Healthcare Diagnostics, USA), with a detection limit of 50 RNA copies/mL. Currently the detection limit is 40 RNA copies/mL (RealTime HIV-1 Assay- Abbott Laboratories). The included patients presented with CD4+ T lymphocytes greater than 400 cells/μL, and no significant alteration was observed between the nadir and current values (Figure 2).

Patient genetic polymorphisms

Genetic polymorphisms associated with slow progression were analyzed, and three out of 22 (14%) patients were heterozygous for the CCR5Δ32 genotype. Receptor use, deduced from the V3 amino acid sequence by genotypic algorithms, was consistent with an R5 phenotype in 21 (95%) of the studied patients.

HIV-1 subtypes

HIV-1 genotyping of the *env* and *pol* genes showed subtype C in 11/22 (50%), subtype B in 7/22 (32%); and the circulating recombinant forms (CRFs) BF in 3/22 (14%) and BC in 1/22 (4%) patients (Figure 3). A recombinant genome in the *pol* region was observed in one sample. Evaluation of the variables according to HIV-1 subtype (B, C, or CRFs BC, or BF) only showed a statistically significant difference ($p = 0.03$) for the variable gender female, with a higher occurrence of subtype C (75% sexual transmission). The remaining variables did not seem to be associated with a specific subtype. Demographic variables and the host and viral characteristics are summarized by HIV-1 subtype in Table 1.

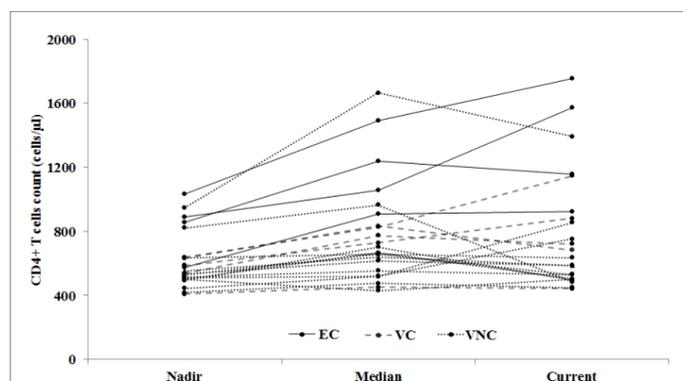
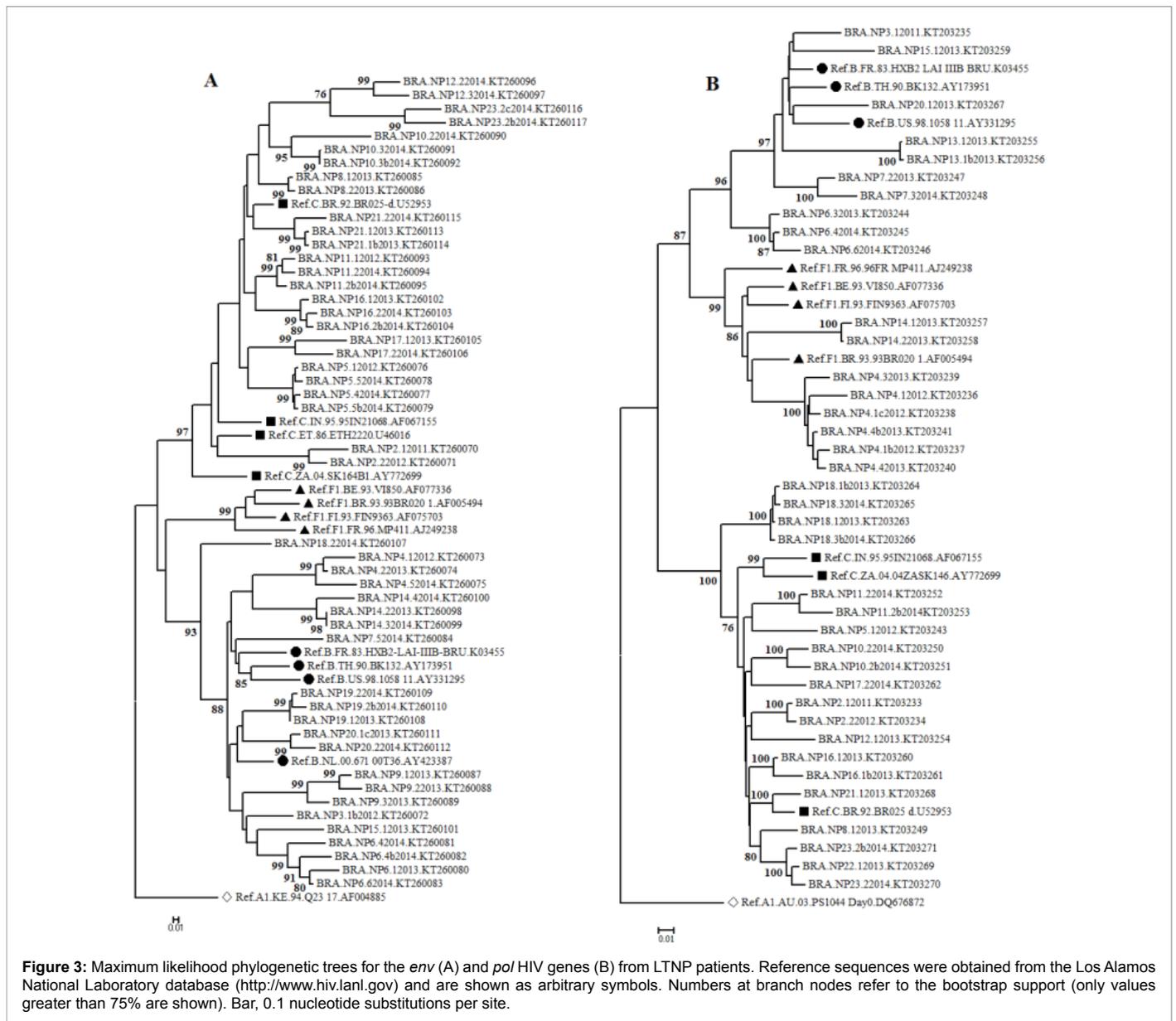


Figure 2: Nadir, median, and current CD4+ T cell counts of elite controllers, viremic controllers, and viremic non-controller HIV patients

Note: *Median*: the median value of all CD4+ T cell counts reported in medical records.

Characteristic	Subtype B N = 7 (%)	Subtype C N = 11 (%)	CRFs N = 4 (%)	Total N = 22 (%)	P value
CCR5Δ32 heterozygous	0 (0)	1 (9)	2 (50)	3 (14)	0.055
Female	1 (14)	8 (73)	3 (75)	12 (54)	0.034
HIV risk factor					
Sexual	4 (57)	6 (55)	4 (100)	14 (63)	0.976
IDU	1 (14)	2 (18)	0 (0)	3 (14)	
Vertical infection	2 (29)	3 (27)	0 (0)	5 (23)	
Median time from HIV-1 diagnosis (IQR)	14y (13-18)	13y (11.5-16)	13y (11.75-14.5)	13.5y (12-16)	0.3438
Subject Age at Diagnosis (Median, IQR)	45y (41-51)	32y (27-37.5)	38.5y (24.75-44.5)	32y (22.5-36.5)	0.3113
LTNP classification					
EC	2/7 (29)	2/11 (18)	0/4 (0)	4 (18)	0.579
VC	1/7 (14)	2/11 (18)	2/4 (50)	5 (23)	0.348
NVC	4/7 (57)	7/11 (64)	2/4 (50)	13 (59)	0.886
Nadir CD4+ T cell count, cells/μL, median (IQR)	633 (489–839)	554 (512–636)	511 (473–544)	545 (501–637)	0.308
EC	874 (865–882)	803 (689–917)	-		
VC	536	636 (635–637)	499 (453–543)		
NVC	538 (436–680)	516 (503–543)	511 (502–520)		
Current CD4+ T cell count, cells/μL, median (IQR)	753 (677–1024)	684(559–1034)	512 (481–618)	702 (530–914)	0.220
EC	1365 (1260–1469)	1337 (1129–1545)	-		
VC	720	915 (799–1030)	661 (551–770)		
NVC	693 (587–787)	588 (517–721)	512 (503–521)		
Viral load, copies/mL, median (IQR)				NA	0.938
EC	Undetectable	Undetectable	-		
VC	1750 (745–6157)	361 (50–949)	724 (162–1948)		
VNC	18873(6814–33039)	9438 (3962–21900)	6980 (3560–11682)		

IDU = intravenous drug user; LTNP = long-term non-progressor; EC = elite controller; VC = viremic controller; VNC = viremic non-controller; CRFs = circulating recombinants forms
Table 1: Patient demographics and viral characteristics of HIV-1-infected long-term non-progressor patients in Curitiba, southern Brazil



Discussion

Although the number of cases included in this study is low, it does not differ from the numbers reported in other studies of LTNPs [2,22,23] since they represent a very select group of patients presenting different patterns to disease progression. HIV-1 LTNP status is supposed to be associated with multiple viral and host factors. However, the mechanisms responsible for this phenotype have not been fully elucidated. Most studies evaluating LTNPs usually include patients with HIV subtype B; this is the first study that shows a cohort of non-progressor patients with different viral subtypes, pointing out that probably host factors likely have a greater correlation with non-progression than viral factors.

We showed a significant predominance of women infected with subtype C. Previous studies conducted in the southern region of Brazilian have already reported that subtype C is more frequently seen in the heterosexual population, with high prevalence of this clade

among women, whereas subtype B is more prevalent in MSM and MSMW [10,24,25]. This association was not observed with other HIV subtypes [16]. This greater predisposition for heterosexual transmission of subtype C appears to be related to a stronger preference for location in the female genital mucosa than other subtypes, which may enable both vertical and heterosexual transmission [5]. No other variables presented an association with a specific HIV-1 subtype; however, this assessment was limited due to the small sample size.

The subtypes found in the study group show the different patterns of HIV-1 subtypes that are distributed in Brazil; whereas the southern region has a higher frequency of subtype C; however, Raboni et al. [10] reported a higher frequency of subtype B (55%), followed by C (30%), F, and the CRFs (both 7.5%) for the city of Curitiba, with approximately the same distributions for gender, age, risk of contamination, and sexual behavior observed in our study. The higher frequency of subtype C observed in this study could be related to the reduced replicative rate

of this subtype. However, more studies are needed to identify any viral changes or mutations that may be related to the defective replication of viruses in this patient group.

HIV-1 subtypes may exhibit differences in transmission rates, disease progression, neurotoxicity, antiretroviral treatment failure profiles, and accuracy of viral load measurements [5]. Several clinical studies have addressed the differences in disease progression and outcome between viral subtypes. A study by Toulomi et al. (CASCADE data) [26] showed that, compared with subtype B, CD4+ T cell decline was significantly slower for subtypes A and CRF02 and marginally slower for subtype C. In addition, in most studies, subtype D was associated with faster disease progression. Easterbrook et al. [27] showed that subtype D was associated with a faster rate of CD4+ T cell decline and a higher rate of treatment failure when compared with subtypes A, B, and C. Other studies showed that compared to other subtypes, subtype D more frequently used the CXCR4 coreceptor, even in the early stages of infection, which could explain the faster rate of CD4+ TC decline and disease progression [28,29]. The relationship between subtype C and disease progression in naïve patients remains unclear. *In vitro* studies suggest that subtype C replication is less competent than subtype B replication [30-32]. In this study, we found that those LTNPs infected with subtype C (VC and NVC) had lower viral loads than those infected with subtype B, although the number of patients in each group was too small to determine the significance of the differences.

Miura et al. [33] showed that control of infection could be established very early, and in LTNPs, it could be the result of infection with attenuated viruses. Several studies have demonstrated the presence of defective viruses in LTNP cohorts [34-36]. In a study of HIV-1 LTNPs by Sandonis et al. [2], major deletions were detected in the 5'-LTR-gag region and *pol* gene that favor virus control, as well mutations in conserved residues in the region from the 5'-LTR to the *nef* gene. Casado et al. [35] reported the presence of low replicating viruses in a set of HIV-1 controllers with rare amino acids in the virus envelope that were associated with the presence of protective host alleles related to HIV control. Host protective factors, such as CCR5-Δ32 heterozygous and protective HLA alleles, have also been shown to be associated with infection control. However, most of these studies were restricted to subtype B virus.

The results of LTNP cohort studies are divergent with regard to the association between CCR5-Δ32 heterozygous and slower progression to AIDS. Concerning CCR5 polymorphisms, the frequencies of the CCR5Δ32 mutation in our study did not differ from that observed among the Caucasian population of seronegative individuals, as previously reported [37,38].

Bioinformatics tools have been widely used to determine coreceptor usage and could provide an alternative approach in clinical practice for screening candidates for CCR5 antagonist therapy, especially in cases where confirmatory assays for coreceptor usage are unavailable [39]. In this study, G2P and Phenoseq analysis showed R5 variants in almost all samples (95%), and the unique patient (VC) who showed the R5/X4 variants was CCR5Δ32 heterozygous. It is possible that other genetic alterations or variations in the expression of specific cellular proteins, such as the restriction factors APOBEC3, TRIM5, and Tetherin, are related to infection control [40]. Studies have shown that LTNPs with viral control tend to have a slightly higher number of CXCR4-expressing cells, although its association with disease progression is not significant [38].

Conclusion

This report is the first study of LTNPs in southern Brazilian, and the data obtained to date aid in our understanding of this group of HIV-infected patients, and will help in the determination of the probable mechanisms associated with delayed clinical progression. As most studies on LTNPs and HIV pathogenesis have focused on subtype B, further studies are needed to elucidate the host-viral interactions in LTNPs with various HIV-1 clades, to clarify the reasons for their slow progression, and to improve our understanding of the factors associated with this phenomenon, which could lead to novel therapeutic interventions.

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