Better CD4+ T Cell Recovery in Brazilian HIV-Infected Individuals Under HAART Due to Cumulative Carriage of SDF-1-3'A, CCR2-V64I, CCR5-D32 and CCR5-Promoter 59029A/G Polymorphisms

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Abstract: Polymorphisms of chemokines and chemokine-receptors genes have been shown to influence the rate of progression to AIDS; however, their influence on response to HAART remains unclear. We investigated the frequency of the SDF-1-3'A, CCR2-64I, CCR5-D32 and CCR5-Promoter-59029-A/G polymorphisms in Brazilian HIV-1-infected and uninfected individuals and their influence on CD4+ T-cell evolution HIV-1 infected individuals before and during HAART. Polymorphism detection was done in a transversal study of 200 HIV-1-infected and 82 uninfected individuals. The rate of CD4+ T cell increase or decrease was studied in a cohort of 155 HIV-1 infected individuals on pre and post-HAART. Polymorphisms were determined by PCR associated with RFLP. The rate of CD4+ T-cell decline or increase was also determined. HIV-1 infected and uninfected subjects showed, respectively, frequencies of 0.193 and 0.220 for SDF-1-3'A, of 0.140 and 0.110 for CCR2-V64I, of 0.038 and 0.055 for CCR5-D32, and of 0.442 and 0.390 for CCR5-P-59029-A/G. HIV-1-infected subjects carrying one, two or three of these four polymorphisms showed better CD4+ T-cell recovery than HIV-1-infected subjects carrying the four wild-type alleles (+2.7, +1.6, +3.5, -0.9 and lymphocytes/µl/month, respectively). Regression logistic analysis showed that the CCR5-D32/CCR2-V64I association was predictor of positive CD4+ T cell slope after HAART. The distribution of polymorphisms did not differ between HIV-1-infected and uninfected individuals, but differed from more homogenous ethnic groups probably reflecting the miscegenation of the Brazilian population. We add further evidence of the role of these polymorphisms by showing that the CD4 gain was influenced by carriage of one or more of the polymorphisms studied here. These results highlight the possibility that these genetic traits can be useful to identify patients at risk for faster progression to AIDS or therapeutic failure.

Keywords: HIV-1, CCR5-D32, SDF-1-3'A, CCR2-V64I, CCR5-PROMOTER-59029A/G, polymorphisms, chemokine receptors.

INTRODUCTION

Chemokines and their receptors play an important role in human immunodeficiency virus type 1 (HIV-1) transmission and pathogenesis [1-5]. The β-chemokine receptor 5 (CCR5) is a coreceptor used by HIV macrophage-tropic strains to enter CD4+ expressing cells. Expression of CCR5 is therefore critical to HIV transmission and progression to AIDS, with R5 strains being isolated preferentially early in infection [6-8]. Another important coreceptor is the α-chemokine receptor 4, CXCR4, a coreceptor used by X4 strains which are preferentially isolated at late stages of the disease [2,8,9]. On the other hand, chemokines behave as potent blockers of the infection of the cell by competing with the virus for receptor binding. The β-chemokines, MIP-1α, MIP-1β and RANTES, are CCR5 ligands while the α-chemokine, SDF–1, is the sole ligand of CXCR4. Several chemokine/chemokine receptor polymorphisms affecting the expression or function of chemokines or their receptors have been shown to impact HIV-1-related disease progression [8-10]. The presence of a 32-bp deletion variant from the coding region of the CCR5 gene (CCR5-D32) leads to the synthesis of a truncated CCR5 protein, which was shown to provide partial protection against HIV-1 infection in individuals homozygote for this genotype (CCR5-D32/D32), as well as protection against disease progression in HIV-infected individuals with the heterozygous genotype [6-7,11-13]. Moreover, several single-nucleotide polymorphisms (SNPs) located in the regulatory region of the CCR5 gene have been reported to be involved in the rate of disease progression to AIDS [14-15]. The SNP on the promoter gene of CCR5, located on CCR5-P-59029A/G, has been reported to delay the progression to AIDS probably through down modulation of CCR5 mRNA production [16-18].

Another genetic variation was described in the CXCR4 ligand gene and identified as an alteration on the 3'untranslated region of SDF-1, named SDF-1-3'A [2,8,19]. The presence of the SDF-1-3'A allele was associated with delay in progression to AIDS [19]. However the effects of SDF-1-3'A remain controversial [10,20] since its beneficial effects were not confirmed in other studies [21]. Another polymorphism associated with slowing of progression to AIDS locates on the CCR2 gene (CCR2-V64I). However its influence in susceptibility to HIV-1 infection has not yet been demonstrated [21-26].

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There is evidence that the mechanism by which the host biological and genetic background protects against HIV disease progression is multifactorial and involves restricted viral entry, preserved cell-mediated immunity, as well as other mechanisms [27]. However, the role of these genetic characteristics on disease progression should be reevaluated in the context of the changing natural history of HIV infection consequent to the blockade of HIV replication by the new antiretroviral drugs. In the present study, we aimed to verify whether carriage of one or more of the CCR5-D32, CCR5-P-59029-A/G, CCR2-V64I and SDF-1-3'A polymorphisms could be related to either slower loss of CD4+ T cells before HAART or faster gain of CD4+ T cells after HAART in HIV-1 infected individuals.

MATERIAL AND METHODS

Subjects

The determination of the frequency of the polymorphisms was performed on a cohort of HIV-infected patients on follow up at our outpatient service. This cohort has been defined in previous studies [28]. From March 2000 to December 2002, all patients matriculated at our outpatient service who were referred to our laboratory to undergo routine exams were included, performing a total of 200 patients. Of these, 179 underwent HAART at some moment of their follow up and 155 (77%) were on regular follow up and participated in the study of disease progression. Age range was 21-68 years (median 37); 66% were male and 34% female. In parallel, polymorphism frequency was determined in 82 adult individuals HIV-1 seronegative recruited from the Medical School and Hospital staffs (age range, 19-65 years (median: 29), 44% male and 56% female). The protocol was approved by the Ethical Committee Board of the Hospital das Clínicas and Institute Adolfo Lutz, and all participants signed an informed consent form.

Leukocyte Separation and DNA Extraction

Whole blood was collected in EDTA anti-coagulant tubes and leukocytes were isolated by incubating with 0.4% saponin for 10 minutes and then washed twice with PBS and stored at -70ºC. The DNA was extracted using the Proteinase K method. Briefly, the cell pellet was incubated for 60 min at 60ºC and for 10 min at 100ºC with a mixture containing a "digest solution" (50 mM KCl and 10 mM Tris-HCl, [Sigma Chemicals, St Louis, Montana, USA], a 5% solution of the detergent Igepal CA-630 [Sigma], 5% Tween-20 [Sigma] and 20 mg/ml Proteinase-K [Gibco BRL, Gaithersburg, Maryland, EUA]). Cell lysates were then stored at -70ºC.

Characterization of SDF-1-3'A, CCR2-V64I, CCR5-D32 and CCR5-P-59029A/G Polymorphisms

Genomic DNA from $1.0x10^6$ cells was amplified by PCR. CCR5-D32 was determined by PCR using the primers: CCR5-D1 5'-AAC/AGA/TCT/CAA/AAA/GAA/GGT/CT-3' and CC R5-D25'-CAT/GAT/GGT/GAA/GAT/AAG/CCT/CAC/A-3' (kindly donated by H. W. Sheppard, CDHS, CA, USA, as previously described [23,29-30]). The PCR conditions were as follows: 1 x PCR buffer [Invitrogen, Carlsbad, California, USA], 3.0mM MgCl2, 10µM of each dNTP, 0.6µM of each primer, and 1 unit of Taq polymerase [Invitrogen]. The cycling scheme was 94ºC for 9 min; followed by 26 cycles of: 94ºC for 30 s, 65ºC for 30 s decreasing 0.5ºC every cycle to 55ºC and 72ºC for 30 s; followed by 15 cycles of 94ºC for 30 s, 55ºC for 30 s and 72ºC for 30 s; followed by 1 cycle of 72ºC for 10 minutes, followed by a hold at 4ºC. After amplification the reaction was electrophoresed through a 1% of agarose gel with 100 bp ladder [Invitrogen, and visualized with ethidium bromide staining]. The CCR5 wild type allele produced a 230 base pair (bp) fragment, while the CCR5-D32 allele produced a 198 bp fragment.

The SDF-1-3'A, CCR2-V64I and CCR5-P-59029A/G polymorphism was determined by PCR using the primers: SDF-1-3-Foward: 5'-CAG/TCA/ACCC/TGG/GCA/AAB/CC-3' and SDF-1-3-reverse AGC/TTT/GGT/CCT/GAG/AGT/C C-3'; CCR2B-reverse 5'TTG/TGG/GCA/ACA/TGA/ TGG-3' and CCR2B-forward 5'CTG/TGA/ATA/ATT/TGC/ACA/ TTG/C-3' and CCR5-Promoter-Foward 5'-CCC/GTG/AGC/ CCA/TAG/TTA/AAA/CTC-3' and CCR5-Promoter-Reverse 5'-TCA/CAG/GGC/TTT/TCA/ACA/GTA/GG-3'. PCR conditions and cycling scheme were the same as described for CCR5-D32 genotyping. After amplification, reactions were digested with MspI, BsaB1 or Bsp1268I [New England Biolaboratories, Ipswich, Massachussetts, USA] to identify, respectively, the CCR2-V64I allele, the SDF-1 wild type allele or the CCR5-P-59029-A/G allele [16,31]. The reaction was electrophoresed as described above. CCR2 amplification resulted in a 183bp product and after digestion the CCR2-V64I allele was identified by the production of two fragments, one of 165 bp and other of 18 bp. After digestion, the SDF-1 PCR product resulted in a fragment of 300 bp, while digestion of the wild type allele PCR product generated two fragments, one of 200 and one of 100 bp; the SDF-1-3'A PCR product digestion generated a fragment of 300 bp. For the CCR5-Promoter, the PCR product was a 268bp fragment; the wild type allele PCR product remained with the same length after digestion while the CCR5-P-59029-A/G was digested into three fragments of 131, 127 and 10 bp.

CD4+ and CD8+ T-cell Phenotyping

Flow cytometry analyses were performed with fresh blood samples collected in tubes with EDTA. Whole blood was stained with $CD4^{FITC}$, $CD8^{PE}$ and $CD3^{PerCP}$ [BD, San Jose, California, USA] for 30 min at room temperature to determine the CD4+ and CD8+ T-cells. Then, red cells were lysated using Multi-Q-Prep and analyzed by means of Coulter EPICs XL-MCL Flow Cytometer [Beckman Coulter, Fullerton, California, USA].

Determination of HIV-1 Viral Load

Plasma viral load was determined using the NASBA Amplicor assay following the manufacturer's instructions [Nuclisens HIV-1 QT, Biomerieux, Durham, North Caroline, USA]. It is worth pointing out that this test only became available at Brazilian Reference Laboratories Network in 1998, when many patients were already under treatment with HAART.

Slope Calculation

The CD4+ T cell, CD8+T cell, and HIV-1 viral load slopes before and after HAART were calculated as the difference in the absolute number between two subsequent determinations divided by the number of days between the two determinations. The average slope was then calculated from the slope obtained for each interval and multiplied by

30 to obtain the monthly variation. Only those patients having more than 11 months of follow up and at least three laboratory determinations were included in this analysis (n = 60 before HAART and n = 155 during HAART use). HIV-1 viral load slope was evaluated for 22 patients before treatment and for 154 patients after treatment initiation.

Statistical Analysis

Allele frequency was determined based on the formula $f = (1 \times h + 2H) / 2N$, as previously described by Luccote [32], where h is the number of heterozygotes, H the number of homozygotes and N the total number of individuals; the frequency of each allele was compared by chi square proportion test. Heterogeneity between population samples was evaluated by chi square of Pearson. The values of CD4/CD8 T-cell and HIV-1 viral load slopes were calculated using Excel 2000 software.

To compare two independent groups the Mann-Whitney test was performed, while the Kruskall-Wallys test with Dunn's post-test was used to analyze three or more groups. The logistic regression model was performed with bivariate data and the candidate variables with a statistical significance of $p<0.10$ were chosen for the final logistical model. Logistic regression models, with calculation of the corresponding crude and adjusted odds ratio and 95% confidence intervals, were used to examine the differences between the CD4+ increase or decrease along time (positive or negative slope).

Level of significance was set at $p<0.05$. The data were analyzed using using GraphPad Prism 3.0 [GraphPad Software, San Diego, California, USA] and SPSS for Windows 10.0 [SPSS, Chicago, ILL, USA].

RESULTS

1. SDF-1-3'A, CCR2-V64I, CCR5-D32 and CCR5-P-59029A/G Allele Distribution in HIV-1 Uninfected and Infected Individuals

SDF-1-3'A, CCR2-V64I, CCR5-D32 and CCR5-P-59029A/G polymorphisms occur at variable frequency according to the ethnic groups [14,17,23,33-38]. Since the Brazilian population, differently from many other populations, is highly mixed, our initial aim was to determine the frequency of these four polymorphisms in HIV-1 infected and uninfected individuals in São Paulo, Brazil. The Hardy-Weinberg Equilibrium has been calculated and revealed that all polymorphisms were in equilibrium, as shown in Table **1**. We found that the allele and genotype frequency of these polymorphisms was similar between HIV-infected and uninfected populations (Table **1**). In general, the frequencies differed from those found in other less ethnically mixed populations [14,17,23,33-39]. The CCR2-V64I, SDF-1-3'A, and specially the CCR5-D32 polymorphisms were as rare in our population as in other populations with less miscegenation, while the CCR5-P-59029A/G polymorphism was relatively frequent, similarly to the other studied populations. Of note, CCR5-D32 homozygosis was not found in our populations. In addition, we analyzed the frequency of the four matched genotypes between the HIV-infected and HIV-uninfected populations, and again we did not find any significant difference (data not shown).

2. Polymorphisms Effects on CD4+ T Cells Variation

Carriage of the SDF-1-3'A, CCR2-V64I, CCR5-D32 and/or CCR5-P-59029A/G polymorphisms confers a protective effect on progression to AIDS in HIV-1 infected individuals [8]. We thus aimed to verify in our cohort of HIV patients whether these polymorphisms were associated with different slopes of the parameters used to monitor the infection along time, such as CD4+ and CD8+ T-cell counts and HIV-1 viral load determination. We calculated the monthly variation of CD4+ T and CD8+ T-cell counts and the HIV-1 viral load in infected individuals classified according to the SDF-1-3'A, CCR2-V64I, CCR5-D32 and CCR5-P-59029A/G polymorphisms, before and after HAART introduction. CD8+ T-cell and viral load variations along time did not show any significant association with carriage of polymorphisms (data not shown). The CD4+ T cell variation before HAART in our cohort was -5.3 (± 14) CD4+ T

Table 1. Allele Frequency of SDF-1-3'A, CCR2-V64I, CCR5-D32 and CCR5-59029^{A}/G in HIV-1 Infected and Uninfected Individuals

Gene Locus	Allele Frequency	HWE	Genotype Frequency			x^2	Odds Ratio (95% CI)
	HWE	*p*	+/+ n (%)	+/- n (%)	-/- n (%)	*p*	
SDF-1-3'A							
HIV-1 infected (n=200)	0.193	0.10	134 (*67*)	55 (*27*)	11 (*6*)	0.40	1.75 (0.71-1.93)
HIV-1 uninfected (n=82)	0.220	0.97	50 (*61*)	28 (*34*)	4 (*5*)		
CCR2-V64I							
HIV-1 infected (n=197)	0.140	0.92	146 (*74*)	47 (*24*)	4 (*2*)	0.45	0.43 (0.43-1.45)
HIV-1 uninfected (n=82)	0.110	0.98	65 (*79*)	16 (*20*)	1 (*1*)		
CCR5-D32							
HIV-1 infected (n=200)	0.038	0.58	185 (*92*)	15 (*8*)	0 (*0*)	0.38	1.46 (0.62-3.48)
HIV-1 uninfected (n=82)	0.055	0.60	73 (*89*)	9 (*11*)	0 (*0*)		
CCR5-P-59029 A/G							
HIV-1 infected (n=200)	0.442	0.06	55 (*28*)	110 (*56*)	32 (*16*)	0.96	1.01 (0.66-1.54)
HIV-1 uninfected (n=82)	0.390	0.49	29 (*35*)	42 (*51*)	11 (*14*)		

HWE – Hardy Wenberg Equilibrium; +/+: homozygous wild type pe to allele, +/-: heterozygous, -/-: homozygous to deletion or polymorphism; *p* and OR refer to the comparison of the genotypes between the infected and uninfected individuals.

cells/µL/monthly (n = 55) and +1.8 (± 5) CD4 + T cells/ µL/monthly (n = 155) after HAART.

2.1. CD4+ T-Cells Monthly Variation Among HIV-1 Infected Individuals Classified by the Presence of SDF-1-3'A, CCR2-V64I, CCR5-D32 and CCR5-P-59029A/G Polymorphisms

We first analyzed each polymorphism separately (Table **2**). Among HIV-1-infected individuals not taking HAART, those heterozygote for CCR2-V64I showed a slightly lower CD4+ T-cell loss than those with wild genotype; this difference however did not reach statistical significance (-3.9 *vs* -9.7 CD4+ T-cell/µl/monthly, p= 0.08). Carriage of the SDF-1-3'A and CCR5-P-59029-A/G polymorphisms did not significantly affect the CD4+ T-cell loss before HAART, while analysis of the CCR5-D32 polymorphism was impaired due to the reduced number of patients (n=9) heterozygous for this allele.

On the other hand, after HAART initiation, HIV-1 infected individuals heterozygote for CCR5-D32 were able to reconstitute better their CD4+ T-cell compartment than CCR5-D32 wild type individuals (+5.0 *vs* +1.7 CD4+ T-cell/µlmm3/monthly, p=.01) (Table **2**). Those heterozygote for, SDF-1-3'A and CCR5-P-59029-A/G polymorphisms did not exhibit a better CD4+ T-cell reconstitution. Finally, the low number of patients homozygote to SDF-1-3'-A and to CCR2-V64I precluded slope analyses. CCR5-P-59029-A/G homozygosis also did not affect CD4 T-cell reconstitution.

2.2. CD4+ T-Cell Monthly Variation Among HIV-1 Infected Individuals Classified by the Presence of Polymorphisms in One or More Genes

We next speculated whether the presence of one or more polymorphic genes, either in heterozygosis or homozygosis, was associated with an accelerated loss of CD4+ T-cell before HAART or with a better recovery after HAART. Analysis of patients before HAART was not feasible due to the low number of patients without any polymorphism found. Among patients on HAART, as shown in Table **3**, carriage of either one, two or three polymorphic gene was associated with positive CD4+ T-cell slopes (+2.3, + 1.6 and +3.5 CD4+ T-cell/µl/month, respectively), while no carriage at all genes was associated with a negative slope (-0.9 CD4+ T cell/µl/month). No patient carrying the four polymorphisms was found in our population.

2.3. Polymorphisms Predictors of a Positive CD4+ T Cell Slope After HAART

The polymorphisms that behaved as predictors of a positive CD4+ T cell slope after HAART, according to logistic regression analyses, were the CCR5-D32 (p=0.001 and odds ratio = 0.228 [CI: 0.153-0972]) and CCR2-V64I (p=0.035 and odds ratio = 0.508 [0.270 a 0.954]). Thus, these analyses provided evidence that the association between CCR5-D32 and CCR2-V64I are predictors for the positive CD4+ T cell slope after HAART.

Table 2. Monthly CD4+ T-Cell Slope (Cells/µl) Before or During HAART of HIV-1 Infected Patients Classified by SDF-1-3'A, CCR2-V64I, CCR5-D32, CCR5-P-59029-A/G (Allele Analysis)

	HAART	SDF-1-3'A		CCR2-V64I		CCR5-D32		CCR5-P59029-A/G	
		Pre	Post	Pre	Post	Pre	Post	Pre	Post
Wild Type	n	42	107	43	116	56	144	14	45
	Mean of time follow up (years)	1.6	5.1	1.6	5.2	1.8	5.1	1.8	4.8
	CD4+ T cell slope (±SD) (cell/µl)	-8.0 (±9.8)	+1.5 (±5.2)	-9.7 (±10.0)	+2.1 (±4.9)	-7.2 (±9.2)	+1.7 (±2.5)	-8.0 (±10.2)	+1.9 (±4.9)
	Median CD4+T cell count at first visit	367	243	386	262	359	228	380	294
	Median CD4+T cell count at last visit	198	367	253	387	271	367	328	408
Heterozygous	n	17	42	19	36	6	11	38	86
	Mean of time follow up (years)	1.6	4.9	1.9	4.7	1.5	4.9	1.8	5.1
	CD4+ T cell slope (±SD) (cell/µl)	-7.6 (±10.1)	+2.5 (±4.1)	-3.9 (±7.6)	+1.4 (±5.1)	- 14.7 (±11.3)	+5.0 * (±7.5)	-8.0 (±9.9)	+2.0 (±4.7)
	Median CD4+T cell count at first visit	339	293	339	211	595	293	274	226
	Median CD4+T cell count at last visit	3.02	347	271	353	270	530	196	387
Homozygous	n	3	6	0	3	0	0	9	23
	Mean of time follow up (years)	1.7	5.1	-	5.8	-	-	1.8	5.3
	CD4+ T cell slope (±SD) (cell/µl)	-7.7 (±5.0)	+5.7 (±5.2)	-	+2.7 (±7.6)	-	-	-7.0 (±9.0)	+1.8 (±6.2)
	Median CD4+T cell count at first visit	430,0	173,0	-	289,0	-	-	274,0	271,0
	Median CD4+T cell count at last visit	356,0	541,5	-	398,0	-	-	196,0	340

Wild type genotype represents individuals in which both alleles are wild type alleles. Heterozygotes are those who carry one polymorphic or mutant allele and one wild type. n = sample number. SD = standard deviation of the mean.

Number of years of follow up did not differ between the respective wild type, heterozygous and homozygous groups (P=0.12 to 0.98, Mann-Whitney or Kruskall-Wallis test).

Median CD4+ T cell count either at first visit or at last visit did not differ significantly between the respective wild type, heterozygous and homozygous groups for each gene/genotype (P = 0.40 to 0.98, Kruskall-Wallis test)

*Heterozygous *vs* Wild Type, p<0.05 (Mann-Whitney Test).

Table 3. Monthly CD4+ T-Cell Slope (Cells/μl), Time of Follow Up (Years) and CD4+ T Cell Count After HAART of HIV-1 Infected Patients Grouped by the Presence of None, One, Two or Three Polymorphisms Considering the SDF-1-3'A, CCR2-V64I, CCR5-D32 and CCR5-P-59029-A/G Genotypes

	n	CD4 + T Cell Slope (Cells/μl)	Δt* (Years)	Median of Last CD4 Before HAART	Median of Last CD4 After HAART
No polymorphic allele (A)	16	**-0.9** (4.2)	4.9	310	367
1 polymorphism (B)	78	**+2.3** (5.2) †	5.6	226	423
2 polymorphisms (C)	51	**+1.6** (4.8) ‡	5.8	298	352
3 polymorphisms (D)	9	**+3.5** (5.9) †	4.1	181	571

(+) = cell gain; (-) = cell loss. All results are shown as means (SD).
Number of years of follow up did not differ between the groups ($P = 0.80$, Kruskall-Wallis test).
Median CD4+ T cell count before HAART did not significantly differ among the four groups ($P = 0.76$, Kruskall-Wallis test).
*Δt: time of follow up for calculation of the slope.
†$P<0.05$ and ‡$P = 0.07$ *vs* A.

DISCUSSION

HIV disease progression is a complex phenomenon reflecting biological and behavioral aspects. Studies on the human genetic background are important to understand the disease natural history and the variations in treatment responses, as well as to plan effective preventive interventions. In Brazil, few studies have been done that exploit the genetic background of HIV-1 infected patients. We studied polymorphisms on the genes of four chemokine system components that have been reported to influence AIDS progression: SDF-1-3'A, CCR2-V64I, CCR5-D32 and CCR5-P59029-A/G [8].

Table 4. Logistical Regression – Polymorphisms Predictors of a Positive Slope of CD4+ T Cells

	Significance p	Odds Ratio	95.0% CI	
			Lower	Upper
CCR5-D32	0.001	0.228	0.153	0.972
CCR2-V64I	0.035	0.508	0.270	0.954

CI: confidence interval.

The frequencies of SDF-1-3'A, CCR2-V64I, CCR5-D32 and CCR5-P-59029A/G alleles vary according to the ethnic group studied [14,17,23,33-39]. Brazilian population is formed by extensive admixture among Amerindians, Europeans and Africans, probably constituting one of the populations with the most varied genetic background in the world [40,41]. The patients were not stratified according to color skin, since recent studies of ancestry-informative markers of the Brazilian population showed that at individual level, color, as determined by physical evaluation, was a poor predictor of genomic ancestry, and genetic evaluation showed that individuals classified by skin color carries genetic markers from Amerindians, Europeans and Africans, concluding that Brazilian population carries a unique genetic background [40,41]. Maybe due to this fact, some differences in allele frequencies compared to other more homogenous ethnic groups were observed in the present study. The SDF-1-3'A allele frequency in HIV-1 infected (0.193) and uninfected (0.220) individuals in our study was similar to that reported in Caucasians (0.221), but higher than that found among Hispanic (0.160) or African Americans (0.057), and lower than among Asians (0.257) [19]. Another Brazilian study reported similar frequencies [42]. CCR2-V64I allele frequency in HIV infected (0.140) and uninfected individuals (0.110) studied here was somewhat higher than in other reports from HIV infected urban populations from Brazil (0.081), but lower than that found among two groups of HIV-uninfected Brazilian Amerindians (0.260-0.300) [43,44]. The CCR2-V64I frequency was higher among Brazilians than Caucasians (0.098) but lower than among African Americans (0.151) [22] or Chinese (0.217) [45]. The CCR5-D32 distribution in our HIV infected (0.038) and uninfected population (0.055) was similar to that in other Brazilian reports (0.035-0.067) [43,46-47], but lower than that found among Caucasian Europeans (0.092) [33].

The CCR5-P-59029A/G polymorphism has not been previously studied among Brazilian individuals. Compared with the other polymorphisms, we found a relatively high frequency of this polymorphic allele in HIV infected (0.442) and uninfected (0.390) populations. However, these frequencies were lower than those seen in North Americans HIV-infected and uninfected individuals, irrespective of their genetic background [16].

We then evaluated the effect of the polymorphic alleles and genes distribution HIV-1 disease progression, before and after HAART use, based on CD4+ and CD8+ T-cell counts and viral loads, all parameters currently employed for monitoring the course of the infection. Although the introduction of HAART has dramatically reduced the development of AIDS by HIV-infected individuals, treatment response varies among patients possibly due to genetic background and environmental factors [10].

When the polymorphic alleles were analyzed individually, CCR5-D32 carriage was associated with improved CD4+ T cell gain during HAART, confirming previous studies showing a protective effect of CCR5-D32 on other markers of disease progression of the HIV-1 infection during HAART [13,21,25,29,52-54]. The CCR5-D32 allele is known to encode a truncated protein that is not expressed on cell surface. In fact, we have recently observed that HIV-1 infected individuals who were heterozygous to CCR5-D32 but otherwise were wild type to the other polymorphisms had down-modulated the CCR5 expression on CD4+ T cell as compared to HIV-1 infected individuals without any polymorphism [Rigato *et al.*, unpublished data].

Through logistic regression analyses it was found that then association between CCR5-D32 and CCR2-V64I was a predictor of positive CD4+ T cell slope after HAART. Our findings corroborate previous data in which CCR2-V64I allele was shown to exert a protector effect in progression of the infection when associated with CCR5-D32 [49,50]. Our data are consistent with the hypothesis that this polymorphism would increase the intracellular expression of CCR2-A isoform, which binds to CCR5, down-modulating its surface expression and possibly restraining HIV infection of the host cells [30,51]. In fact, CCR2-V64I allele presence has been associated with the slowing of the progression to AIDS in the pre-HAART setting [21-22,46].

On the other hand, we were not able to detect any effect due to the presence or absence of SDF-1-3'A and CCR5-P-59029-A/G polymorphisms. In fact, data on SDF-1-3'A is rather controversial. SDF-1-3'A homozygosis has been either associated to an accelerated progression to AIDS, but with a subsequent prolonged survival after AIDS diagnosis [31], or to a marked slowing in progression to AIDS and to death [19]. This issue was further reviewed by Ioannidis *et al*. who concluded that SDF-1-3'A does not have a role in disease progression [21]. Our data suggest that, if a protective effect does exist, it is not associated with better CD4+ T-cell recovery. Possibly, this polymorphism may play a major role at later phases of the infection, when the circulating viruses have the X4 phenotype [55], and can find more unbound CXCR4 receptors due to the lower levels of its natural ligand, SDF-1, in SDF-1-3'A homozygous individuals [56-58]. Consistent with our results on CCR5-P-59029A/G, previous reports on this polymorphism either disclosed only a relative protection [16] or no effect on AIDS progression of patients on HAART [59].

However, the strength of these individual polymorphism analyses, including that one for CCR2-V641 after HAART, might have been weakened by the fact that the groups carrying each of these alleles were compared to individuals who did not carry the respective allele, but who could carry any one of the three remaining polymorphisms, including the CCR5-D32 allele that confers some protection. Thus, they were compared with "control" groups that already may carry some degree of protection. Other limitation of the study was that viral genotyping was not performed. Concerning patients' adherence, our cohort was previously characterized as having a 67% rate of good compliance (among those with available information), being irregular in only 33% [28].

Next, we studied the effect of the cumulative presence of potentially protective chemokine and their receptors polymorphisms on the slopes of CD4+ and CD8+ T cells and viral load variations over time, before and after treatment. We verified that HIV infected patients carrying one to three of four polymorphism (SDF-1-3'A, CCR2-V64I, CCR5-D32, or CCR5-P-59029-A/G) had a better reconstitution of CD4+ T-cell during HAART. Carriage of four polymorphisms could not be evaluated due to absence of individuals with such background. These results reinforce the notion that individual's background influences the natural history of the disease, even in the setting of HAART, which otherwise represents a strong "environmental" pressure, encompassing these subtle genetic influences and tending to render the patients' treatment responses equal [48]. Our data are also in agreement with the major role played by CD4 T-cell counting, but not CD8 T-cell counting or HIV-1 viral load for the long term follow up of patients since we could not find any important association between the polymorphisms carriages and the slopes of CD8+ T-cell and viral load (data not shown).

In summary, as the pivotal role of β-chemokines and SDF-1 and their receptors CCR5 and CXCR4 on HIV entry in the host's cells has come up, one could speculate that polymorphisms in the genes encoding these and possibly other molecules of the chemokine system would influence the natural history of the disease. Previous studies have so far demonstrated this influence by analyzing several parameters such as time to AIDS, time to death, survival curves, among other characteristics. Our study adds further evidence for the role of these polymorphisms by showing that the CD4 gain was influenced by carriage of one or more of the polymorphisms studied here. Our preliminary data also suggest that the individuals among our cohort who were wild type to CCR5-D32 and SDF-1-3'A evolved more frequently to death than those carrying a polymorphism in these genes [Rigato *et al*., unpublished data]. Overall, these results highlight the possibility that these genetic traits can be useful to identify patients at risk for progressing faster to AIDS or developing therapeutic failure. Moreover, with the advent of new drugs for treating HIV patients, such as CCR5 inhibitors that act by blocking HIV entry, it will become increasingly important to determine the genotype of the patients, since CCR5-D32, CCR5-P59029-A/G and/or CCR2-V64I carriage may result in poorer treatment responses.

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