Analysis of Immunological, Viral, Genetic, and Environmental Factors That Might Be Associated with Decreased Susceptibility to HIV Infection in Serodiscordant Couples in Florianópolis, Southern Brazil

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Abstract

Individuals who have been exposed to human immunodeficiency virus (HIV) and have not been infected might possess natural resistance mechanisms. An understanding of the sociodemographic and immunological conditions that influence resistance to HIV is a challenge, and very little is known about the role of intrinsic antiviral factors that restrict HIV infection. The aim of this study was to analyze potential factors responsible for resistance to HIV infection in serodiscordant couples by comparing HIV-exposed seronegative individuals (HESN) to HIV-seropositive individuals treated with antiretroviral therapy (HIV-ART) along with healthy controls (HC). The results revealed one HLA-B*27 and two HLA-B*57 individuals among the HESN; a CCR5Δ32 heterozygous deletion was observed in one serodiscordant couple, while the homozygous genotype for this variant was not observed. There were no differences in the basal mRNA expression of APOBEC3G, CFLAR, TRIM5α, LEDGF/p75, BST-2, or SAMHD1 in CD4⁺ T lymphocyte- and monocyte-enriched populations among the three groups, and lower HBD-3 concentrations were observed in saliva from HIV-ART compared to HESN and HC. The most prevalent HIV-1 subtype was C or C-containing recombinant forms. Six HIV-ART individuals and one HIV-ART individual were infected with the R5 HIV and X4 HIV strains, respectively. The ability to control infection or delay disease progression is probably defined by a balance between viral and host factors, and further evaluation should be performed in larger cohorts. Our data suggest that susceptibility to HIV infection varies among individuals and strengthens the multifactorial characteristics underlying the resistance mechanisms in HIV.

Introduction

EXPOSURE TO HUMAN IMMUNODEFICIENCY VIRUS (HIV) does not always result in infection. Currently, there are many reports of individuals who have been exposed to HIV and do not exhibit clinical or serological evidence of infection, suggesting the existence of mechanisms that lead to natural resistance. These individuals are known as HIV-exposed seronegative (HESN) subjects and can be represented by serodiscordant couples; individuals with high-risk sexual behaviors, such as commercial sex workers and men who have sex with men; and individuals who are exposed via nonsexual routes, such as injection drug users, infants born to HIV-infected mothers, hemophiliacs, and others who are exposed to

contaminated blood products.¹ There is currently no consensus regarding the factors that are responsible for protection in HESN. These factors are certainly multifactorial and include contributions from the host, such as immunological and behavioral features and the genetic background, as well as from the virus, such as viral tropism, load, and subtype.

At present, more than 80% of the infections worldwide are transmitted by sexual intercourse, and the study of serodiscordant couples, generally monogamous couples in which only one partner is infected, is considered relevant for the identification of factors associated with protection. Studies investigating serodiscordant couples have previously described some factors associated with protection among HESN, such as the presence of the 32-base-pair (bp) deletion

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in chemokine CC receptor 5 (CCR5 Δ 32),² differences in T cell reactivity to HIV,³ and the presence of cationic peptides in cervicovaginal secretions.⁴ Although Brazil has the largest number of HIV-infected people among the countries in Central and South America,⁵ and an ethnically very diverse population,⁶ there is a paucity of information regarding cohorts of serodiscordant couples. The few reports on this subject in Brazil have addressed genetic polymorphisms in *CXCL12*⁷ and *CCR5*,² the presence of specific *HLA* alleles,⁸ patterns of activation in CD4⁺ T and CD8⁺ T lymphocytes,⁹ sexual transmission profiles,¹⁰ or sociodemographic characteristics associated with HIV infection.⁹ Reports evaluating cellular host proteins that interact with HIV during its replication cycle have not yet been published.

AIDS-related mortality in Southern Brazil is steadily increasing and, since the beginning of the twenty-first century, is highest among the Brazilian regions. In 2013, the incidence in Santa Catarina State, one of the three states in Southern Brazil, was 32.2 cases per 100,000 inhabitants, which is of the largest among the Brazilian states.¹¹ Moreover, the HIV epidemic in this region presents a different profile compared to the rest of the country because it demonstrates a cocirculation of HIV subtypes B and C at similar frequencies.¹² Together with a reasonable health care system from an HIV care perspective, this feature makes Southern Brazil an important site for epidemiological studies that could promote a better understanding of the natural resistance to HIV-1 infection.

The aim of the present study was to correlate the multiple immunological, viral, genetic, and environmental factors that might be involved in natural resistance to HIV infection. To address this issue, we compared sociodemographic features, sexual behavior, and immunological and genetic characteristics in a cohort of Brazilian HIV serodiscordant couples (HESN) from Florianópolis, Santa Catarina State, consisting of HIV-seropositive individuals who were treated with anti-retroviral therapy (HIV-ARV) and a control group of healthy controls (HC). In addition, we evaluated some features of the virus in HIV-seropositive partners.

Materials and Methods

Ethics statement

The Universidade Federal de Santa Catarina Ethics Committee approved the study. All of the study subjects provided written informed consent prior to enrollment.

Study population

This study was conducted in a cohort of heterosexual and homosexual couples aged greater than 18 years. The couples were in a stable relationship for at least 12 months, discordant for HIV infection, and had an episode of virus exposure through unprotected sexual intercourse. They were recruited at reference centers in Florianópolis, Brazil, from June to December 2012. Saliva and blood samples were collected from nine serodiscordant couples (nine HESN and nine HIV-ART) and 12 HC who were seronegative for HIV. HESN and HC status was confirmed by serological and nucleic acid test (NAT) assays. Clinical patient data were obtained from the medical records, and exclusion criteria were a positive serological test for hepatitis C virus or clinical manifestations of Epstein-Barr virus, cytomegalovirus, herpesvirus type 1,

or herpesvirus type 2. Male condom provision and sexual risk reduction counseling were provided during every interview.

Sample collection and processing

Saliva production was stimulated by applying a drop of 2% citric acid in the sublingual region and collecting spit in a tube containing Protease Inhibitor Cocktail (Sigma-Aldrich). This procedure was repeated three times at 30-s intervals, the samples were centrifuged at 2,800 $\times$ g for 10 min, and the supernatant was stored at -80°C . From each subject, a peripheral blood sample (30 ml) was collected into EDTA tubes, and the plasma and peripheral blood mononuclear cells (PBMCs) were separated using the Histopaque (Sigma-Aldrich) density gradient method. CD4⁺ T lymphocytes and monocytes were isolated from PBMCs using the RosetteSep Human CD4⁺ T Cell Enrichment Cocktail and RosetteSep Human Monocyte Enrichment Cocktail kits (Stemcell Technologies), respectively. The cell purity was determined using fluorochrome-labeled anti-CD3, anti-CD4, and anti-CD14 antibodies (BD Pharmingen), a FACSCalibur flow cytometer (Becton Dickinson), and the software FlowJo 8.6.3 (Tree Star). The samples were stored in RNAlater, and total RNA was isolated using the PureLink RNA Mini Kit (Ambion), according to the manufacturer's instructions. Genomic DNA from PBMCs was extracted using the QIAamp DNA Blood Kit (Qiagen).

HLA genotyping

HLA class I genotyping was performed using the polymerase chain reaction sequence-specific oligonucleotide (PCR-SSO) method combined with Luminex technology with an SSO-LABType HLA class I (HLA-A, -B, and -C) commercial kit (One Lambda, Inc.) according to the manufacturer's instructions. In this method, genomic DNA was amplified using a biotinylated sequencing primer locus specific for HLA class I in a GeneAmp PCR System 9700 thermocycler (Applied Biosystems). Subsequently, hybridization was performed with complementary DNA probes conjugated to beads that were labeled with different fluorochromes to identify complementary sequences of the amplified DNA. After hybridization, the samples were read using the flow cytometry platform LABScanTM100 (One Lambda, Inc.) followed by analysis with HLA Fusion software version 2.0 (One Lambda, Inc.).

CCR5 Δ 32 genotyping

Genomic DNA was extracted from PBMCs using a resin column procedure (Biopur, Biometrix Diagnóstica) according to the manufacturer's protocol. The *CCR5* gene was amplified by PCR, and a fragment consisting of 225 bp was obtained using the following primers: sense 5'-ACC AGA TCT CAA AAA GAA 3' and antisense 5' CAT GAT GGT GAA GAT AAG CTT CA 3' (Invitrogen, Life Technologies). Briefly, 100 ng of DNA, 2.5 μM forward and reverse primers, 1.25 μM dNTP mix (Invitrogen Life Technologies), 50 μM MgCl₂, and 2 units of Taq DNA polymerase (Invitrogen Life Technologies) were subjected to 35 PCR cycles, each consisting of denaturation at 94 $^{\circ}\text{C}$ for 1 min, annealing at 58 $^{\circ}\text{C}$ for 1 min, and extension at 72 $^{\circ}\text{C}$ for 1 min. Predenaturation and further extension were performed at 94 $^{\circ}\text{C}$ for 5 min and 72 $^{\circ}\text{C}$ for 10 min, respectively. PCR was performed in a

thermocycler (Applied Biosystems Veriti 96-Well Thermal Cycler, Life Technologies). The PCR products were analyzed by electrophoresis in a 3% agarose gel and visualized using an ultraviolet transilluminator after staining with 1% ethidium bromide. The wild-type and variant alleles produced 225-bp and 193-bp fragments, respectively. The electrophoresis profile was captured and recorded using the photo documentation system L-PIX-HE (Loccus Biotecnologia).

β -Defensin quantification

The β -defensins HBD2 and HBD3 were quantified in saliva and plasma using a commercial sandwich enzyme-linked immunoassay (ELISA, PeproTech). Briefly, 96-well plates (BD Pharmingen) were coated with 5 μ g anti-HBD2 or anti-HBD3 antibodies per well at room temperature for 16 h. Blocking was conducted with 1% bovine serum albumin in phosphate-buffered solution at room temperature for 1 h. Next, 100 μ l of the samples was added to each well and maintained at room temperature for 2 h, followed by the addition of 2.5 μ g of secondary antibody for 2 h. To each well was added 1 μ g of streptavidin-peroxidase (Sigma-Aldrich), and the samples were maintained at room temperature for an additional 30 min. A further incubation was conducted with 2,2'-azino-bis-3-ethylbenzthiazoline-6-sulfonic acid (Sigma-Aldrich) diluted in citrate buffer (citric acid 0.05 M plus 0.01% H_2O_2) in the dark at room temperature for 10 min. The absorbance was measured at 415 nm with a microplate reader (Infinite M200, Tecan). Recombinant HBD2 and HBD3 were used as positive controls and to generate the standard curve.

Gene expression analysis

The mRNA expression analysis was performed by reverse transcriptase quantitative polymerase chain reaction (RT-qPCR). Complementary DNAs (cDNA) were synthesized from 160 ng of total DNase-treated RNA using the SuperScript III First-Strand kit (Invitrogen, Life Technologies) according to the manufacturer's instructions. Primers for target-specific and endogenous reference genes were obtained from published reports^{13–16} or designed using the Primer3 Input program (www.bioinformatics.nl/cgi-bin/primer3plus/primer3plus.cgi/) with the help of the CLC Sequence Viewer 6.8.2 program and assessed with the PrimerBlast tool (www.ncbi.nlm.nih.gov/tools/primer-blast/) available in GenBank (Supplementary Table S1; Supplementary Data are available online at www.liebertpub.com/aid). The relative gene expression was measured using the 7900 HT Fast Real Time PCR System (Applied Biosystems, Life Technologies) and SYBR Green PCR Master Mix (Applied Biosystems, Life Technologies).

The cycling program consisted of 50°C (2 min) and 95°C (10 min), followed by 40 cycles of 95°C (15 s) and 60°C (60 s). Melting curve analysis was performed after the completion of qPCR to collect fluorescence values between 60°C and 95°C at 0.5°C increments. The qPCR data were analyzed using the ABI 7900HT Sequence Detection System Software version 2.4 (Applied Biosystems, Life Technologies). For the standard curve, the cDNAs of Human Reference Total RNA (Clontech) were used. The results (C_q values) were exported, and outliers with C_q values demonstrating a difference of more than 1.0 were excluded. The C_q values of four different reference genes, *beta 2 microglobulin (B2M)*, *ribosomal protein L13a (RPL13A)*, *TATA box binding protein (TBP)*,

and *ubiquitin C (UBC)*, were analyzed using a program available online (www.leonxie.com/referencegene.php?type=reference) and selected to normalize the target gene expression.

The ΔC_q data matrix was used to calculate the relative expression (fold change) using the $2^{-\Delta\Delta C_q}$ method (assuming 100% amplification efficiency).¹⁷ Various fold-change and raw C_q cut-offs were applied to assess the value of the sensitivity of the RT-qPCR assay. The delta C_q values (ΔC_q) were determined for all of the target genes by subtracting the C_q values from the geometric means of the C_q values for the three reference genes. The amplicon length of the PCR product was validated by 2.5% agarose DNA gel electrophoresis, and the PCR products were visualized with 0.1% ethidium bromide versus a 100-bp DNA ladder (NorGen).

Analysis of subtype and viral tropism

The HIV-1 envelope (*env*) (relative to HXB2 6846–7360) and polymerase (*pol*) (relative to HXB2 2274–3545) were amplified by nested PCR from PBMC genomic DNA using primers and reaction conditions as previously described.¹⁸ The purified PCR products were sequenced using BigDye Terminator v3.1 Cycle Sequencing (Applied Biosystems, Life Technologies) and analyzed with an ABI 3100 Genetic Analyzer (Applied Biosystems, Life Technologies). The generated sequences were then assembled and visually inspected using SeqMan software in the LaserGene package (DNASTAR). The HIV-1 subtype was assigned by submitting *env* and *pol* sequences to the REGA, RIP, and SCUEAL online tools.^{19–21} Recombination analysis was performed with the bootscanning method as implemented in SimPlot software.²² For the *in silico* prediction of HIV tropism, *env* sequences were translated into amino acids and submitted to the Geno2pheno online tool.²³ Subtype C sequences were additionally submitted to analysis in CoRSeqV3-C, a tool that has been specially developed to predict HIV-1 subtype C tropism.²⁴

Statistical analysis

Statistical analyses were performed using SPSS version 17.0. The sample normality was tested using the Shapiro–Wilk test. Differences between individuals in the different study groups were investigated with the Mann–Whitney *U*-test or Student's *t*-test. The comparison of three or more variables was performed using the Kruskal–Wallis test or “one-way” analysis of variance (ANOVA) with Dunnett's post hoc analysis. The frequencies obtained for the categorical variables were compared using Fisher's exact test. Observations were considered statistically significant at $p < 0.05$.

Results

Study population

Nine serodiscordant couples (HESN and HIV-ART) and 12 HC were enrolled in this study. The main characteristics of the population are shown in Table 1. Among the serodiscordant couples, eight (88.9%) were heterosexual and one (11.1%) was a man who had sex with other men. HESN and HIV-ART were more often males (55.6%). Eight of the serodiscordant couples (88.9%) were in a steady relationship for more than 2 years. The average age of the HESN and HIV-ART individuals was 38.7 and 41.7 years, respectively. Four

TABLE 1. GENERAL CHARACTERISTICS IN THE STUDY POPULATION

	HESN (n=9)	HIV-ART (n=9)	HC (n=12)
Age (years $\pm$ SD)	38.7 $\pm$ 9.0	41.7 $\pm$ 9.2	35.9 $\pm$ 12.6
Gender (% male)	5 (55.6%)	5 (55.6%)	6 (50.0%)
Relationship duration (% two years or more)	8 (88.9%)	8 (88.9%)	7 (58.3%)
Body weight (% overweight)	4 (44.4%)	2 (22.2%)	6 (50.0%)
Education degree (% complete high school)	1 (11.1%)	0	5 (41.7%)
Income (% stable employment status)	5 (55.6%)	4 (44.4%)	12 (100.0%)
Family income (US\$ average)	<1,100	<1,100	>2,000
Tattooed individuals	1 (11.1%)	4 (44.5%)	3 (25.0%)
Rest (% sleep less than necessary)	6 (66.7%)	5 (55.6%)	5 (41.7%)
Alcoholic beverages consumption (% weekly)	2 (22.2%)	1 (11.1%)	1 (11.1%)
Fried foods consumption (% weekly)	7 (77.8%)	6 (66.7%)	5 (41.7%)
Red meat consumption (% weekly)	9 (100.0%)	8 (88.9%)	8 (66.7%)
Fruits and vegetables consumption (% weekly)	6 (66.7%)	7 (77.8%)	11 (91.7%)
Cereals consumption (% weekly)	3 (33.3%)	0	9 (75.0%)
Physical exercise (% weekly)	5 (55.6%)	3 (33.3%)	5 (41.7%)
Smoking (% smokers)	4 (44.4%)	3 (33.3%)	0
Blood transfusions	1 (11.1%)	2 (22.2%)	0
Illicit drugs (% users)	2 (22.2%)	4 (44.4%)	0
Diagnosis (years $\pm$ SD)	Not applicable	9.2 $\pm$ 4.9	Not applicable
CD4 ⁺ T cell count (cells/ml $\pm$ SD)	Unavailable	519 $\pm$ 287.5	Unavailable
HIV viral load	Undetectable	Undetectable	Undetectable
Condom use in oral sex (% never)	8 (100%)	8 (100%)	7 (58.3%)
Condom use in sex with vaginal penetration (% never)	7 (77.8%)	7 (77.8%)	10 (83.3%)
Condom use in anal sex (% never)	6 (66.7%)	6 (66.7%)	6 (50.0%)

Data are average values or percentages, if indicated.

SD, standard deviation; HESN, HIV-exposed seronegative; HIV-ART, antiretroviral therapy-treated HIV-infected subjects; HC, healthy controls.

(44.4%) HESN and two (22.2%) HIV-ART individuals were overweight. Regarding education level, only one (11.1%) individual from the HESN group reported completion of high school, while four (44.4%) reported at least 8 years of school. In contrast, none of the HIV-ART individuals had completed high school, and seven of them (77.8%) reported at least 8 years of school.

Concerning income, in the HESN group, five (55.6%) individuals reported a stable employment status, three (33.3%) were retired, and one (11.1%) had no occupation. In the HIV-ART group, four (44.4%) people had a stable employment status, two (22.2%) were retired, and three (33.3%) had no occupation. The average monthly income of the couples was less than US\$ 1,100. Considering the control group, the average age of the HC individuals was 35.9 years.

The HC subjects were in a monogamous and heterosexual relationship for at least 1.5 year at the time of enrollment, and seven (58.3%) were in relationships for more than 2 years. Seven (58.3%) had at least 8 years of study and five (41.7%) had completed high school. Six (50.0%) individuals in the HC group were overweight, and all of the members in this group had stable employment with an average family monthly income greater than US\$ 2,000. Tattooed individuals were present in all groups, with a higher frequency in the HIV-ART group, in which four (44.5%) subjects had tattoos.

Most participants reported sleeping less than necessary and ingesting alcoholic beverages, fried foods, red meat, vegetables, and fruits at similar frequencies, while regular cereal consumption was higher in the HC group. Physical exercise was less common in the HIV-ART group. Finally, blood transfusions, smokers, and illicit drug users were observed only among serodiscordant couples. The periodontal condition

of the serodiscordant couples was evaluated through Periodontal Screening and Recording (PSR) to exclude the possibility that variations in the concentration of defensins in the saliva were linked to periodontal diseases. One (11.1%) person in the HESN group presented with gingivitis (score 2), and all of the other evaluated individuals had good periodontal health (score 0).

All of the subjects reported low percentages of condom use. Serodiscordant couples reported not using condoms during oral sex (one couple reported not performing oral sex), and only one couple reported the use of condoms during all episodes of vaginal or anal penetration (common among serodiscordant couples). The practice of unprotected sex was also common among individuals in the HC, in which the majority of them did not use condoms during oral or vaginal sex. In addition, three HC who reported practicing anal sex did not use condoms. ANOVA revealed no differences in comparisons of serodiscordant couples and HC. HIV-ART individuals were diagnosed at an average of 9.2 years. The average CD4⁺ T cell count in the HIV-ART group was 519.3 cells/mm³, and the RNA HIV viral load was undetectable in all subjects.

HLA genotyping

HLA genotyping analyses revealed the presence of class-I alleles associated with protective effects. *HLA-B*27* was observed in one (11.1%) and *HLA-B*57* in two (22.2%) HESN (Table 2). However, *HLA-B*57* was also present in two (22.2%) HIV-ART. *HLA-B*14* and *HLA-C*08*, protective-related alleles, were observed in one (11.1%) HIV-ART individual. Furthermore, one (11.1%) HIV-ART presented

TABLE 2. HUMAN LEUKOCYTE ANTIGEN (HLA) GENOTYPING OF HIV-SERODISCORDANT COUPLES

HIV-serodiscordant couple	HESN			HIV-ART		
	HLA-A	HLA-B	HLA-C	HLA-A	HLA-B	HLA-C
1	A*02 A*31	B*35 B*39	C*04 C*07	A*01 A*32	B*40 B*57	C*02 C*06
2	A*02 A*68	<u>B*44</u> B*51	C*01 C*05	A*02 A*03	B*07 B*39	C*07 C*07
3	A*23 A*31	B*44 B*52	C*04 C*15	A*02 A*02	Ambiguous	C*03 C*07
4	A*24 A*29	B*52 B*57	C*15 C*18	A*24 A*68	B*18 B*52	C*05 C*12
5	A*01 <u>A*03</u>	B*08 B*27	C*02 C*07	A*03 A*32	<u>B*14</u> B*35	C*04 C*08
6	A*01 A*25	B*18 B*50	C*06 C*12	A*01 A*11	<u>B*18</u> <u>B*37</u>	C*06 C*07
7	A*02 A*33	<u>B*35</u> B*57	C*04 C*06	A*11 A*11	<u>B*52</u> B*57	C*07 C*12
8	A*02 A*24	<u>B*39</u> B*44	C*05 C*07	A*02 A*24	NA	C*05 C*07
9	A*01 A*02	B*08 <u>B*35</u>	C*01 C*07	A*02 A*24	<u>B*35</u> B*40	C*03 C*04

Protective-related alleles are in *italic* and potential disease progression-related alleles are *underlined*.

HESN, HIV-exposed seronegative; HIV-ART, antiretroviral therapy-treated HIV-infected subjects; NA, not assayed.

homozygosity for the *HLA-A*02* allele. Unexpectedly, *HLA-A*29* was detected in one (11.1%) and *HLA-B*35* in one (11.1%) HESN. These alleles might potentiate disease progression similar to *HLA-B*18*, which was observed in two (22.2%) HESN and one (11.1%) HIV-ART. However, information about disease progression was not available because the individuals were not followed up after blood collection.

CCR5 genotyping

The presence of the *CCR5Δ32* allele was evaluated in all individuals. The *CCR5Δ32* homozygous deletion (*Δ32/Δ32*) was absent in all individuals. The *CCR5Δ32* heterozygous deletion (*CCR5/Δ32*) was observed in two (22.2%) HESN subjects, two (22.2%) HIV-ART patients, and one (8.3%) HC. The DNA sample from one HC individual was degraded and thus was not adequate for CCR5 genotyping. Table 3 shows the presence of the *CCR5Δ32* allele among members of serodiscordant couples and the viral tropism found in HIV-ART individuals.

β-Defensin quantification

The HBD2 mean concentrations $\pm$ standard error in the HC, HIV-ART, and HESN groups were, respectively, $0.36 \pm$

0.07 ng/ml, 0.26 ± 0.06 ng/ml, and 0.16 ± 0.03 ng/ml in plasma and 2.35 ± 0.18 ng/ml, 2.25 ± 0.29 ng/ml, and 2.15 ± 0.33 ng/ml in saliva. There were no significant differences between the obtained values. The mean concentrations of HBD3 in HC, HIV-ART, and HESN were, respectively, 0.47 ± 0.07 ng/ml, 0.38 ± 0.07 ng/ml, and 0.46 ± 0.07 ng/ml in plasma and 58.04 ± 9.89 ng/ml, 28.96 ± 10.73 ng/ml, and 55.03 ± 11.56 ng/ml in saliva. In the three groups, a higher amount for HBD3 compared to HBD2 was observed in plasma and saliva. The saliva concentration of HBD3 in HIV-seropositive partners was significantly lower than that in HC and seronegative partners ($p=0.01$) (Fig. 1).

Basal mRNA of APOBEC3G, CFLAR, TRIM5α, LEDGF/p75, BST-2, and SAMHD1 in CD4⁺ T lymphocyte- and monocyte-enriched populations

All of the primers used in the RT-qPCR reactions showed very good specificity, which was confirmed by the presence of a single peak in the dissociation curve. The reaction efficiencies were between 96.9% and 99.3% (data not shown). The C_q values for the four endogenous reference genes were analyzed using a program available online (www.leonxie.com/referencegene.php?type=reference), and the geometric mean of the more stable genes was used for normalization. In

TABLE 3. CCR5Δ32 GENOTYPING, HIV SUBTYPE, AND TROPISM IN HIV-SERODISCORDANT COUPLES

HIV-serodiscordant Couple	CCR5 genotype ^a		HIV subtype		Viral tropism
	HESN	HIV-ART	HIV-ART		HIV-ART
	(Ethnicity)	(Ethnicity)	(env)	(pol)	
1	CCR5/Δ32 (Euro-descendant)	CCR5/Δ32 (Euro-descendant)	B	NA	CXCR4
2	CCR5/Δ32 (Euro-descendant)	CCR5/CCR5 (Afro-descendant)	C	C	CCR5
3	CCR5/CCR5 (Euro-descendant)	CCR5/CCR5 (Euro-descendant)	NA	C	NA
4	CCR5/CCR5 (Euro-descendant)	CCR5/CCR5 (Euro-descendant)	B	BC	CCR5
5	CCR5/CCR5 (Euro-descendant)	CCR5/CCR5 (Euro-descendant)	B	CFB	CCR5
6	CCR5/CCR5 (Afro-descendant)	CCR5/Δ32 (Euro-descendant)	NA	C	NA
7	CCR5/CCR5 (Afro-descendant)	CCR5/CCR5 (Euro-descendant)	C	C or BC	CCR5
8	CCR5/CCR5 (Euro-descendant)	CCR5/CCR5 (Euro-descendant)	B	B	CCR5
9	CCR5/CCR5 (Euro-descendant)	CCR5/CCR5 (Indigenous-descendant)	B	BC	CCR5

^aCCR5/CCR5, wild-type genotype; CCR5/Δ32, heterozygous genotype for the deletion; Δ32/Δ32, homozygous genotype for the deletion. HESN, HIV-exposed seronegative; HIV-ART, antiretroviral therapy-treated HIV-infected subjects; NA, not assayed.

FIG. 1. β -Defensin quantification in saliva and plasma. HBD2 and HBD3 β -defensins were quantified in saliva and plasma samples from healthy controls (HC) (●), antiretroviral therapy-treated HIV-infected subjects (HIV-ART) (■), and HIV-exposed seronegative individuals (HESN) (▲) using an enzyme-linked immunoassay (ELISA). The points show the concentration of HBD2 and HBD3 β -defensins in each individual, while the dotted line denotes the mean concentration and the vertical trace marks are the standard error in each group. * $p < 0.05$.

the CD4⁺ T lymphocyte-enriched population, the geometric mean of *B2M*, *RPL13A*, *TBP*, and *UBC* was used; for the monocyte-enriched population, the geometric mean of *B2M*, *TBP*, and *UBC* displayed a higher stability.

In the present study, there were no significant differences in the basal gene expression of host proteins that interact with HIV during its replication cycle, such as the transcriptional cofactor of HIV-integrase named lens epithelium-derived

growth factor (*LEDGF/p75*) and restriction factors such as apolipoprotein B mRNA editing enzyme catalytic polypeptide-like 3G (*APOBEC3G*), tripartite motif 5 alpha (*TRIM5 α*), bone marrow stromal cell antigen 2 (*BST-2*), CASP8 and FADD-like apoptosis regulator (*CFLAR*), and SAM domain and HD domain-containing protein 1 (*SAMHD1*) in monocyte- and in CD4⁺ T lymphocyte-enriched populations of HESN, HIV-ART, and HC (Fig. 2).

FIG. 2. Basal mRNA levels of *APOBEC3G*, *BST-2*, *CFLAR*, *SAMHD1*, *TRIM5 α* , and *LEDGF/p75*. (A) CD4⁺ T lymphocyte- and (B) monocyte-enriched populations in the antiretroviral therapy-treated HIV-infected subjects (HIV-ART), HIV-exposed seronegative (HESN), and healthy controls (HC). Data are average C_q values transformed into $2^{-\Delta C_q}$.

Analysis of subtype and viral tropism

Among nine HIV-1 isolates analyzed, *pol* and *env* sequences were generated for seven patients. However, the sequences of at least one of the genes were obtained for all nine patients, among which four (44.4%) were classified as subtype C, two (22.2%) as B/C intersubtype recombinants, two (22.2%) as subtype B, and one (11.1%) as the B/C/F mosaic form (Table 3). Subtype C sequences were amplified from individuals with heterosexual exposure and injection drug users, while subtype B was identified in two individuals with heterosexual exposure. The B/C intersubtype recombinants were found in the only representative of a man who has sex with other men. Moreover, infection with the B/C/F mosaic form was observed in a heterosexual individual. The prediction of viral tropism was accomplished based on the sequences of the viral envelope (gp120 C2–V3 region) and enabled the elucidation of seven of the nine HIV-ART individuals. Six individuals were infected with R5 HIV strains and one was infected with X4 HIV strains (Table 3).

Discussion

The aim of this transversal study was to characterize host and viral factors in HIV serodiscordant couples in steady relationships that may lead to a decreased susceptibility to HIV infection. Few studies characterizing HIV-1 serodiscordant couples have been conducted in Brazil, and some of the analyses presented herein are unprecedented in the Brazilian population. In the present cohort, the age and male:female ratio were similar among the three groups. The most striking sociodemographic feature was that compared with HC, seropositive individuals had the lowest income, in addition to a poor education and unhealthy habits such as poor diet, smoking, and illicit drug use. These characteristics are consistent with those reported in similar studies performed in Brazil, Africa, and China.^{9,25,26}

HESN showed a trend toward increased consumption of red meat and fried foods and lower consumption of fruit, vegetables, and cereals. Although most people were not overweight, this condition was less frequent in the HIV-ART group compared with the other two groups. In comparison with the beginning of the AIDS epidemic, the prevalence of overweight people has increased among those living with HIV/AIDS, but the proportion of excess weight found in this study is lower than that found in the healthy Brazilian population.²⁷

Condoms were rarely used in oral, anal, or vaginal sex by any of the individuals; a low frequency of condom use was also demonstrated in another Brazilian study.²⁸ In addition, four (44.4%) of the HIV-ART individuals were diagnosed during their relationship only after showing clinical symptoms, an indication that HESN was exposed to high viral loads for some time. The transmission and acquisition of HIV-1 have been associated with viral and host biological characteristics as well as viral load. In the present study, all of the HIV-ART individuals had an undetectable viral load because they were undergoing treatment with antiretroviral therapy.

The most commonly used drugs (six of nine patients) were efavirenz in combination with lamivudine and zidovudine. The following associations were also being used: efavirenz plus lamivudine and didanosine, efavirenz plus lamivudine

and tenofovir, with one patient using darunavir, raltegravir, lamivudine, ritonavir, and tenofovir. Currently, some studies of serodiscordant couples have shown that antiretroviral therapy can significantly reduce the sexual transmission of HIV.^{29–31} Despite these encouraging results, condom usage should always be promoted, even in patients with viral suppression who may have a detectable viral load in body fluids other than the blood.³² It is important to note that some individuals were undergoing treatment with drugs indicated for the management of therapeutic failure, and three of the HIV-ART individuals (33.3%) exhibited low CD4⁺ T cell counts (lower than 350 cells per mm³), providing evidence of a previous high viral load.

Considering the genetic characteristics of the host, HLA genotyping was performed. Independent cohort studies have demonstrated the effects of specific HLA class I alleles on the rate of progression to AIDS with acceleration conferred by *HLA-A*29*, *-B*22*, *-B*35*, and *-C*16*.³³ Most notably, a number of alleles have been associated with nonprogression to AIDS or reduced susceptibility to HIV infection, such as *-B*14*, *-B*27*, *-B*57*, *-C*08*, and *-C*14*. In Brazil, a study comparing the presence of HLA alleles in long-term non-progressors to disease showed an increased frequency of *HLA-B*52* in resistant individuals and no differences in the frequencies of *HLA-B*27* and *HLA-B*57*.⁸ In the present study, *HLA-B*52* was not observed in any of the HESN, but *HLA-B*27* was observed in one and *HLA-B*57* in two HESN. However, an association among HLA alleles and host and viral characteristics could not be assessed due to the small sample size of the present cohort.

Another genetic factor that was assessed was the presence of *CCR5Δ32*, which is the best characterized host restriction allele associated with protection against HIV-1 infection. Homozygous individuals for the *CCR5Δ32 allele* (*Δ32/Δ32*) have been found to be resistant to HIV-1 infection, while heterozygotes (*CCR5/Δ32*) exhibit a slower disease progression compared with those who are homozygous for *CCR5*.³⁴ In the present study, the *Δ32/Δ32* genotype was not observed, although compared with the results of other studies in Brazil, our results indicated a higher proportion (more than 20%) of the *CCR5/Δ32* genotype in HESN and in HIV-ART as well as in HC individuals (9%). These reports demonstrated a *CCR5/Δ32* frequency of 6.8% among white individuals, 3.8% among blacks, and 6.4% among brown individuals in HC from the city of Alegrete, Rio Grande do Sul.³⁵

One study investigating serodiscordant couples showed a frequency of approximately 9.0% of *CCR5/Δ32* in HESN, 11.0% in HIV-seropositive asymptomatic people, and 6.0% among HIV-seropositive people with AIDS. In addition, approximately 0.06% of the *Δ32/Δ32* genotype was observed among HESN, HC, and HIV-seropositive asymptomatic people, and less than 0.03% of the *Δ32/Δ32* genotype was detected among HIV-seropositive people with AIDS.² The *CCR5/Δ32* deletion is prevalent in European populations, demonstrating a wide variation of 10–20%, and it is almost absent in Asian groups.³⁴ The results obtained in the present study may reflect the high European immigration rate in Southern Brazil.²

Other host characteristics, such as the presence of β -defensins and intracellular proteins, were evaluated. HBD2 and HBD3 β -defensins are capable of preventing HIV penetration in PBMCs³⁶ and inhibiting viral replication

in vitro.³⁷ Our results did not show increased levels of β -defensins in HESN. A study in Kenya reported a similar result for the levels of β -defensins in the cervicovaginal secretions of sex workers.³⁸ However, a Colombian study showed increased levels of β -defensin mRNA in HESN.³⁹ Still, significantly reduced amounts of HBD3 were detected in the saliva in HIV-ART. Lower concentrations of HBD3 were also observed in the placenta of HIV-seropositive mothers compared with HIV-seronegative mothers.⁴⁰ Other reports have noted changes in antimicrobial peptides when saliva samples from HIV-ART subjects were evaluated and showed that the levels of HBD2 in saliva seemed to increase and decrease with the short-term and long-term use of antiretroviral therapy, respectively.⁴¹ Thus, while β -defensins are well characterized as capable of interfering with the cycle of HIV *in vitro*, further investigations of their interactions with HIV *in vivo* are necessary.

HIV interacts with many cellular host proteins during its replication cycle. Some of these proteins exhibit antiviral activity, such as APOBEC3G, TRIM5 α , BST-2, CFLAR, and SAMHD1, while others are required for HIV replication and act as viral cofactors, such as LEDGF/p75.⁴² There were no significant differences in the gene expression of APOBEC3G, BST-2, CFLAR, LEDGF/p75, SAMHD1, or TRIM5 α in monocyte- or CD4⁺ T lymphocyte-enriched populations from HESN, HIV-ART, or HC. Our results are consistent with others that also showed no differences in the expression of these genes among serodiscordant couples.¹⁶ However, some studies have demonstrated an increase in the expression of APOBEC3G in HESN when compared with HC^{13,43} and an increase in SAMHD1 mRNA levels in the CD4⁺ T lymphocytes of elite controllers compared with healthy individuals.⁴⁴ Moreover, two important features of this report must be considered. First, we used four reference genes, while most reports use only one reference gene, and this strategy may support the improved stability and reliability of our results. Second, we analyzed the basal mRNA of APOBEC3G, CFLAR, TRIM5 α , LEDGF/p75, BST-2, and SAMHD1 in CD4⁺ T lymphocytes or monocytes from enriched subpopulations, while in most reports this evaluation was performed using PBMCs.

In the present study, an analysis of vital factors that may influence HIV infection, such as subtype and tropism, was also conducted. The most prevalent HIV-1 subtype in Brazil is B, while in the Southern region there is a greater prevalence of subtype C.¹² Our results were consistent with this pattern, and no significant associations between the HIV-1 subtype and other host and virus features were observed. The prediction of viral tropism was accomplished based on the sequences of the viral envelope and demonstrated that six HIV-ART individuals were infected with R5 HIV strains and one individual was infected with X4 HIV strains. The rapid depletion of CD4⁺ T lymphocytes is associated with X4 viruses.⁴⁵ Previous reports have observed that subtype B viruses more frequently change tropism to X4 when compared with subtype C.⁴⁶ The only X4 strain observed in the present study was an HIV-1 subtype B strain, reinforcing previous findings. It is interesting to note that the patient infected with the X4 strain was also heterozygous for the CCR5 Δ 32 deletion, which might suggest the occurrence of viral adaptation to the host organism.

The mechanisms by which HIV-1 infection fails in HESN are certainly diverse. Our data suggest that susceptibility to

HIV infection varies among individuals and contribute to our understanding of the multifactorial characteristics that contribute to resistance mechanisms in HIV, as it was not possible to determine a specific factor underlying resistance to infection in seronegative individuals who were exposed to HIV. These preliminary analyses should be extended to a prospective longitudinal study to better identify the protective mechanisms in the ethnically diverse Brazilian population. Furthermore, we could not find any distinctive features among the factors analyzed in the only couple of men who have sex with men.

Since this group is still considered among the highest at risk of HIV infection, we should consider conducting a study including only these individuals. However, the results obtained in the present study may be useful for documenting the predominant sociodemographic and epidemiological characteristics of HIV-1 infection in HIV serodiscordant couples in stable relationships, who are not usually the target of public health interventions for the reduction of HIV transmission. We hope that these data will contribute to the improvement of current politics regarding the prevention and monitoring of HIV infection, as well as new therapeutic programs targeting this population.

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Author Disclosure Statement

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