

## HLA-C Single Nucleotide Polymorphism Associated with Increased Viral Load Level in HIV-1 Infected Individuals from Northeast Brazil

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**Abstract: Background:** Genetic variations in Human leukocyte antigen C (*HLA-C*), Zinc ribbon domain containing 1 (*ZNRD1*) and its antisense RNA (*ZNRD1-ASI*) genes are known to influence the HIV-1 replication and disease progression.

**Objective and Method:** We evaluated the distribution of *HLA-C* (rs10484554, rs9264942) and *ZNRD1* (rs8321) and *ZNRD1-ASI* (rs3869068), single nucleotide polymorphisms (SNPs) in 266 HIV-1-infected and 223 unexposed-uninfected individuals from Northeast Brazil and their relation to HIV-1 infection, CD4 T cells count and viral load pre-treatment.

**Results:** *HLA-C* SNPs were in Linkage Disequilibrium ( $D' = 0.84$ ), constituting four possible haplotypes. Our results showed that *HLA-C*, *ZNRD1* and *ZNRD1-ASI* SNPs as well as *HLA-C* haplotypes frequencies were not significantly different between HIV-1-infected and unexposed-uninfected individuals. In addition, we analyzed *HLA-C* and *ZNRD1* and *ZNRD1-ASI* SNPs considering CD4+ T cell counts and viral load before the antiretroviral treatment. Individuals carrying *HLA-C* rs9264942 TT genotype showed a significant increased level of HIV-1 viral load pre-treatment, in comparison with individuals carrying the CC genotype ( $p$ -value = 0.0092). Finally, we stratified our findings according to *CCR5Δ32* allele presence along with the studied SNPs: no statistically significant influence over viral load pre-treatment has been found.

**Conclusion:** The association between *HLA-C* rs9264942 SNP and viral load prior treatment in an admixed population from North East Brazil was in agreement with findings from previous studies obtained on different ethnic groups; however more studies should be conducted in order to clarify how *HLA-C* impair the HIV-1 replication.

**Keywords:** *HLA-C*, *ZNRD1*, *ZNRD1-ASI*, SNPs, HIV-1 infection, viral load.

### 1. INTRODUCTION

During the evolutionary process, hosts and pathogens are involved in a continuous adaptive struggle, in which the host develops several immunological strategies for pathogens' elimination, while the pathogens develop mechanisms to escape host response. This is the case of HIV-1 infection, since the virus takes advantage of immune host cells for its replication, while the host has a series of restriction factors, known to be under genetic control, that interfere with the virus' ability to replicate [1-3].

So far, two different strategies have been developed to analyze the influence of host genome in the control of HIV infection and virus replication: the first one employed few selected candidate genes, encoding for protein with potential HIV-1 restriction action; the second strategy was based on genome-wide association studies (GWAS) performed on greater number of subjects [4, 5].

When merging the findings from these two different approaches, the HLA locus (6p21) had the strongest influence with HIV-1 post-infection control [4]. Interestingly, the 32 base-pairs (bp) deletion on C-C motif chemokine receptor 5 gene (*CCR5Δ32* allele, 3p21.31) is the only variant that has been associated and successfully replicated in different case control studies as involved in HIV-1 infection control [6].

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Within variants present at HLA locus, those located at *HLA-B* in the region encoding for the peptide-binding groove were significantly associated with regulation of HIV-1 replication [7]. Moreover, the rs9264942 SNP, located at *HLA-C* gene was associated with HIV-1 infection and replication control in different independent GWAS, namely the Euro-CHAVI [8, 9], the International HIV Controller Study [10] and the PRIMO/ANRS [4]. In the latter study, it was in linkage disequilibrium with the *HLA-C* rs10484554 variant, which was also associated with HIV-1 control [11].

In the context of HLA HIV-infection and replication control studies, it has been reported that HIV-1 creates an ideal micro-environment that favors escape from host immune response by *HLA-A*, *HLA-B* and *HLA-C* downregulation [12, 13].

HLA-C expression on cell membrane could protect HIV-1 from the attack by natural killer cells expressing killer Ig-like receptors (KIR). However, HLA-C high levels can increase the antigen presentation to cytotoxic T lymphocytes (CTL), interfering in HIV-1 infection [14, 15]. Recently, it has been reported that HIV-1 is able to downregulate *HLA-C* through Vpu *in vitro*, consequently diminishing HLA-C capacity to restrict CTLs and to suppress viral replication in CD4+ T cells. HIV-1 Vpu has also been described as not able to affect *HLA-A* and *HLA-B* expression [12].

*ZNRD1* (zinc ribbon domain-containing 1) gene, located at 6p21.3 (near the *HLA* locus) and its antisense RNA gene (*ZNRD1-AS1*), have been described as two of the 250 HIV-1 dependency factors required for viral replication [16]. The absence of *ZNRD1* protein was correlated with increased HIV-1 restriction, reducing the R5 or X4-tropic HIV-1 replication [17]. A role in the modulation of susceptibility to HIV-1 infection and disease progression was also ascribed to *ZNRD1* [18, 19].

Several independent GWAS and case-controls studies reported that single nucleotide polymorphisms (SNPs) in *HLA-C*, *ZNRD1-AS1* genes could influence susceptibility to HIV-1 infection and AIDS progression in different groups of individuals, most of them of European ancestry [9, 17-24].

Since the genetic background influences single-gene and GWAS association studies, and no research has been published on Brazilian populations, we analyzed four selected *HLA-C*, *ZNRD1* and *ZNRD1-AS1* SNPs, having been associated with HIV-1 post-infection control in independent replica studies, in HIV-1-infected and unexposed-uninfected individuals from Northeast Brazil in the context of susceptibility to HIV-1 infection, CD4+ T cell counts and viral load levels. Finally, aimed at evaluating the potential synergistic effect of pre-treatment *CCR5* 32 bp deletion with *HLA-C*, *ZNRD1* and *ZNRD1-AS1* SNPs in the HIV-1 outcome, we included the *CCR5*Δ32 variant in our analyses.

## 2. MATERIALS AND METHOD

We enrolled 266 HIV-1-infected and 223 unexposed-uninfected individuals, from Recife metropolitan region (Pernambuco, Brazil); Pernambuco population has been described as an admixture of European (59.7%), African (23%) and Amerindian (17.3%) ancestries [25].

HIV-1-infected and unexposed-uninfected individuals (blood donors negative for HBV, HCV, HTLV-1, HIV-1, syphilis and Chagas disease) were recruited at Institute of Integral Medicine of Pernambuco Professor Fernando Figueira (IMIP) and Institute of Hematology and Hemotherapy of Pernambuco State (HEMOPE), respectively. The Ethics Committees from IMIP (registration n° 2629-13) and HEMOPE (registration n° 00880313.0.00005208) approved the studies.

The HIV-1-infected individuals reported to be infected *via* sexual intercourse, presented no significant co-infections (no syphilis, HCV or HTLV-1 infections, for example) and were vaccinated against HBV or otherwise spontaneously cured from a past infection (no chronic HBV infection cases have been detected in this cohort). The individuals did not report intravenous drug abuse. Although, we do not have information regarding how long the HIV-1-infected individuals have been infected, the median time between the date of diagnosis and treatment start date was 11 months (interquartile range, IQR=1-44.5), and median pre-treatment CD4+ T cell counts (median=380, IQR=231-579.5), indicating that the individuals were in chronic infection stage, infected long enough to be close to the 350 CD4+ T cells/mm<sup>3</sup> threshold, which was previously used in this cohort to indicate antiretroviral treatment start. (Table 1) reports information obtained by analysis of HIV-1-infected individuals' medical reports, regarding sex, age, and pre-treatment CD4+ T cell counts and plasma viral load (pVL).

Genomic DNA was extracted from peripheral blood using the Genomic Prep DNA Isolation Kit<sup>®</sup> (Promega, Madison MD).

*HLA-C* (Entrez ID 3107), *ZNRD1* (Entrez ID 30834) and *ZNRD1-AS1* (Entrez ID 80862) SNPs were selected based on independent GWAS results [9, 17-24]. The selected SNPs were: *HLA-C* rs10484554 C>T (TaqMan<sup>®</sup> genotyping assay C\_29612773\_20), associated with higher HIV-1 pVL in Europeans [11]; *HLA-C* rs9264942 C>T (TaqMan<sup>®</sup> genotyping assay C\_29901957\_10) associated with lower HIV-1 pVL in Europeans [9] and HIV-1 pVL set-point in Han Chinese [26]; *ZNRD1-AS1* rs3869068 C>T (TaqMan<sup>®</sup> genotyping assay C\_26544924\_10) associated with faster CD4+ T cells loss [8] and lower pVL in Europeans [18]; *ZNRD1* rs8321 A>C (TaqMan<sup>®</sup> genotyping assay C\_2437466\_10) found more frequently in European long term non-progressors [5, 21]. SNPs genotyping was performed on ABI 7500 real time PCR platform (Thermo Fisher), using allelic-specific probes (TaqMan).

*CCR5*Δ32 polymorphism was detected as described in Silva-Carvalho *et al.* [27]. Briefly, conventional PCR products were visualized with 3% agarose gel electrophoresis with ethidium bromide staining, allowing the discrimination between the *CCR5* wild-type (184 bp) and Δ32 allele (152 bp).

The conformity to Hardy-Weinberg Equilibrium was evaluated through Chi-square test ( $\chi^2$ ), using Genotype Transposer software [28]. Linkage disequilibrium (LD) and haplotype frequencies were estimated with Haploview software version 4.2 [29].

**Table 1. Characteristics of the study population.**

Characteristic	Unexposed-uninfected Individuals (n=223)	HIV-1-Infected Individuals (n=266)
Sex		
Male, n (%)	60 (26.9)	74 (27.8)
Female, n (%)	163 (73.1)	192 (72.2)
Age in years, mean±SD	29.8 ± 12.3	36.4 ± 8.8
Pre-treatment plasmatic viral load in log10 copies/mL, median (median absolute deviation) [interquartile range]	-	4.29 (0.73) [3.51-4.93]
Baseline CD4+ T cells count in cells/mm <sup>3</sup> , (median absolute deviation) [interquartile range]	-	380 (175) [231-579.5]

*HLA-C*, *ZNRD1* and *ZNRD1-AS1* alleles, genotypes and haplotype frequencies were compared in HIV-1-infected and unexposed-uninfected individuals using the Exact Fisher Test. Odds ratios (OR) and 95% confidence intervals (CI95%) were calculated using the most frequent alleles and haplotypes in controls as reference. Bonferroni correction for multiple comparisons was applied to genetic association tests: p-values ≤ 0.0125 were considered statistically significant. The interaction of *CCR5Δ32* allele with *HLA-C*, *ZNRD1* and *ZNRD1-AS1* genotypes over HIV-1 pVL was assessed through multiple linear regression. Statistical analyses were performed with R software 3.1.2 [30].

Moreover, with the aim of confronting the allele frequencies of the SNPs observed in our study in HIV-1-infected and unexposed-uninfected individuals with those of the general population from Pernambuco, we inferred the allele frequencies (indicated as *f* in the equation below, while *p* stands for proportion) for each polymorphism based on the equation presented in Suarez-Kurtz *et al.* [31], which is:

$$f_{\text{Brazil}} = (f_{\text{Portugal}} * p_{\text{European}}) + (f_{\text{Angola+Mozambique}} * p_{\text{African}}) + (f_{\text{Guarani}} * p_{\text{Amerindian}})$$

Basically, the equation calculates a weighted average of the allele frequency observed in the parental populations of Brazil, considering the proportion of each ancestry found in the Brazilian population that one want to know the frequency. Since we have no data of the populations described in the equation, we used, as proxies, the findings of some related populations that has available data present in 1000 Genomes Project database (<http://browser.1000genomes.org>).

### 3. RESULTS

*HLA-C* (rs10484554, rs9264942), *ZNRD1* (rs8321) and *ZNRD1-AS1* (rs3869068) SNPs frequencies in HIV-1-infected and unexposed-uninfected individuals were in Hardy-Weinberg equilibrium. All SNPs frequencies were not significantly different between HIV-1-infected and unexposed-uninfected individuals, after Bonferroni correction (p-value>0.0125) (Table 2).

*HLA-C* SNPs were in linkage disequilibrium (LD) ( $D' = 0.84$ ), constituting four possible haplotypes (C|T, C|C, T|C and T|T). The haplotype distribution showed no significant differences between HIV-1-infected and unexposed-uninfected individuals (p-value>0.05) (Table 3).

In a pair-wise comparison of median pVL and *HLA-C* rs9264942 genotypes, we found that TT genotype carriers bore higher pVL (median=4.16; IQR = 2.76-4.92) than CC ones (median = 2.87; IQR=0.00-4.10; p-value= 0.0092; Wilcoxon test). These results persisted statistically significant after correction for multiple comparisons (p-value < 0.0166, Table 4). No differences were detected when comparing pVL of heterozygous CT genotype (Medians = 4.06; IQR=2.22-4.72) with CC or TT homozygous genotypes of rs9264942. We found no significant influence of any *HLA-C* (rs10484554, rs9264942), *ZNRD1* (rs8321) or *ZNRD1-AS1* (rs3869068) SNPs and genotypes over baseline CD4+ T cell counts.

*CCR5Δ32* allele was only detected in heterozygotes in our sample. The allele frequency was of 3% (6.1% of heterozygotes) in the HIV-1-infected and 4.5% (9% of heterozygotes) in the unexposed-uninfected individuals, but without significant differences between the groups (p-value=0.26). Therefore, *CCR5Δ32* allele presence along with the other SNPs also did not exert statistically significant influence over pVL (data not shown).

For each polymorphism, we obtained the allele frequencies from European Iberian (IBS), African Yoruba (YRI) and Peruvian South Americans (PEL) genome databases and combined the frequencies considering the admixture of the Pernambuco population, whose genome is resulting from European (59.7%), African (23%) and Amerindian ancestries (17.3%) as described in Coelho *et al.* [25]. We observed that the frequencies do not differ more than 5% with the exception of the rs10484554. The estimated frequency of the T allele for this SNP is 8%, whilst we found a frequency of 16% in our study.

**Table 2. Allelic and genotypes frequencies of SNPs in *HLA-C*, *ZNRD1* and *ZNRD1-AS1* genes in HIV-1-infected and unexposed-uninfected individuals from Northeast Brazil**

Genes/SNPs	Unexposed-uninfected Individuals n (%)	HIV-1-Infected Individuals n(%)	Fisher's Exact Test OR (95%CI), p-value
<i>HLA-C – rs10484554</i>			
C	364 (83.9)	447 (88.3)	Reference
T	70 (16.1)	59 (11.7)	0.69 (0.46-1.01), 0.057
CC	155 (71.4)	200 (79.0)	Reference
CT	54 (24.9)	47 (18.6)	0.67 (0.42-1.08), 0.090
TT	8 (3.7)	6 (2.4)	0.58 (0.16-1.96), 0.413
<i>HLA-C – rs9264942</i>			
T	262 (62.4)	328 (65.0)	Reference
C	158 (37.6)	177 (35.0)	0.89 (0.68-1.18), 0.450
TT	77 (36.7)	111 (44.0)	Reference
CT	108 (51.4)	106 (42.0)	0.68 (0.45-1.03), 0.058
CC	25 (11.9)	35 (14.0)	0.97 (0.52-1.84), 1.000
<i>ZNRD1-AS1 – rs3869068</i>			
C	295 (80.6)	395 (75.4)	Reference
T	71 (19.4)	129 (24.6)	1.36 (0.97-1.91), 0.073
CC	121 (66.1)	145 (55.3)	Reference
CT	53 (29.0)	105 (40.1)	1.65 (1.08-2.55), 0.019
TT	9 (4.9)	12 (4.6)	1.11 (0.41-3.10), 1.000
<i>ZNRD1 – rs8321</i>			
A	416 (97.6)	489 (98.2)	Reference
C	10 (2.4)	9 (1.8)	0.76 (0.27-2.12), 0.645
AA	203 (95.3)	240 (96.4)	Reference
AC	10 (4.7)	9 (3.6)	0.76 (0.27-2.13), 0.641

Significant p-value (p<0.0125)

**Table 3. HLA-C haplotypes frequencies in HIV-1-infected and unexposed-uninfected individuals from Northeast Brazil.**

Haplotypes		Unexposed-uninfected Individuals n (%)	HIV-1-Infected Individuals n (%)	Fisher's Exact Test OR (95%CI), p-value
rs10484554	rs9264942			
C	T	256 (61.2)	310 (63.8)	Reference
C	C	94 (22.5)	118 (24.3)	1.04 (0.74-1.44), 0.871
T	C	62 (14.8)	55 (11.3)	0.73 (0.48-1.11), 0.128
T	T	6 (1.4)	3 (0.6)	0.41 (0.07-1.96), 0.312

Significant p-value (p&lt;0.05)

**Table 4. Median values, and their respective first and third quartile (interquartile), of log<sub>10</sub> viral load in HIV-1-infected individuals, prior to antiretroviral treatment, according to the genotype of the studied polymorphisms.**

Gene - SNP	Median (Interquartile)			p-value*
	CC	CT	TT	
<i>HLA-C</i> – rs10484554 (C/T)	3.91 (2.13-4.73)	4.16 (0.00-4.76)	3.67 (3.36-4.30)	0.9099
<i>HLA-C</i> – rs9264942 (C/T)	2.87 (0.00-4.10)	4.06 (2.22-4.72)	4.16 (2.76-4.92)	0.0331
<i>ZNRD1-ASI</i> – rs3869068 (C/T)	3.91 (1.81-4.64)	3.91 (2.40-4.73)	4.35 (3.10-4.83)	0.7485
	AA	AC	CC	
<i>ZNRD1</i> – rs8321 (A/C)	3.85 (1.90-4.59)	4.84 (3.52-5.14)	-	0.0523

\*p-values of the Kruskal-Wallis Test

#### 4. DISCUSSION

This study conducted in a Brazilian population from North East aimed to evaluate if there were any association between SNPs in genes that interfere in the HIV-1 cell entrance with HIV-1 pre-treatment pVL and CD4+ T cell counts. The allele frequencies of the investigated SNPs, seem to be in agreement with the model we exposed. The difference between the expected and the observed minor allele frequencies ( $\Delta$ ) was more than 5% only for the rs10484554 SNP (it reached 8%), meaning that our population is indeed admixed.

Our findings concerning the association of *HLA-C* rs9264942 CC genotype (p-value = 0.0092) with lower pVL levels when compared to other genotypes are similar to those observed by Thörner *et al.* [18] and Thomas *et al.* [15] in Danish and European-American ancestry cohorts, respectively. The *HLA-C* rs9264942 SNP is located around 35 kb upstream region of *HLA-C* locus; even if its relation with membrane *HLA-C* expression has not been described, previous reviews by Kulpa and Collins [14] and Zipeto and Beretta [13] hypothesized that *HLA-C* overexpression could enhance the antigen-presentation capacity of CTLs, thus,

controlling HIV-1 replication. However, considering the study of Kulkarni *et al.* [32] we can also hypothesize that the effect of -35 SNP rs9264942 on *HLA-C* expression and consequent viral load control, is due to the linkage disequilibrium with another SNP, the rs67384697, that being located in a miR-148a binding site would be the true responsible for the modulation of *HLA-C* expression control. Since the two *HLA-C* variables are in linkage disequilibrium, our results associating the rs9264942 SNP with the control of HIV-1 viral load could reflect a hitchhiking effect of this SNPs, being the rs67384697 SNP the true responsible for *HLA-C* expression modulation.

Other studies reported associations between *HLA-C* variations and HIV-1 replication control in different populations [8, 15, 23, 24, 33-35]: our findings are consistent with the literature and highlight a novel association between an *HLA-C* genetic variant and lower pVL levels in a Northeastern Brazilian population.

When considering *ZNRD1* and *ZNRD1-ASI* SNPs, our findings were not in agreement with the literature. An *et al.* [19] reported a 35% decreased chance of HIV-1 infection when investigating high-risk behavior, uninfected individu-

als in the presence of a haplotype block ( $D'=0.99$ ) formed by 12 *ZNRD1* SNPs, including rs8321 (A allele) and *ZNRD1-ASI* rs3869068 (C allele). This finding was obtained on a Unites States (USA) group of individuals, with a genetic background different from the North East Brazilians considered in the present study. This difference in the genetic ancestries could, at least partially, explain the discrepancies found; in fact, the frequencies of rs8321 A allele and rs3869068 C allele reported in the 1000 Genomes for the CEU (Utah residents with Northern and Western European ancestry from the CEPH collection) population (representing to the US-Europeans) have different values when compared to the North East Brazilian ones (rs8321 A allele CEU 0.89 vs 0.976 in North East Brazilians; rs3869068 C allele CEU 0.94 vs 0.806 in North East Brazilians).

Other reports described the *ZNRD1-ASI* (rs3869068) and *ZNRD1* (rs1048412, rs16896970) gene variants as involved in pVL control in European individuals from EURO-Chavi cohort and Spain [8, 17], CD4+ T cells accelerated depletion in US-European/US-African subjects [19] and progression to AIDS in European and Han Chinese patients [21, 22]. These findings have not been replicated in the North East Brazilian population. Also in this case the differences in the distribution of rs3869068 SNP frequencies when compared to the North East Brazilian population considered in our study could account the different association results obtained (rs3869068 allele frequencies in our study C=0.806, T=0.194; CEU C=0.94, T=0.06; IBS C=0.87, T=0.13; CHB (Han Chinese in Beijing, China) C=0.81, T=0.19; ASW (African ancestry in Southwest USA) C=0.73, T=0.27).

So, while for *HLA-C* rs9264942, our results replicated previous findings obtained in populations with different ethnic background but all showing comparable allelic frequencies for the SNP analyzed, in the case of *ZNRD1* rs8321 and *ZNRD1-ASI* rs3869068, the ancestry different distribution of the two SNPs alleles could be related to the contrasting results characterizing our study involving a highly admixed population such as the North East Brazilians.

Moreover, we are aware of the two main limitations of our study: the first one concerns the relative low number of subjects (both HIV-1-infected and unexposed-uninfected individuals) enrolled. The second one is the lack of a proper control group to evaluate the impact of the host genome of susceptibility to HIV-1 infection; in fact the best control group would be exposed-uninfected individuals, however at present time enrolling a group of HIV-1 exposed-uninfected individuals is quite difficult, since mother-to-child transmission has been drastically reduced in Northeast Brazil (where HIV-1-infected and unexposed-uninfected have been recruited) reaching percentages around 1%, and HIV-1 serodiscordant partners are extremely rare. Being so rare the cases of HIV-1 exposed-uninfected individuals, it has been impossible to enroll a sufficient number of individuals for a basic association study as the one presented here.

The control of HIV-1 infection is a multifactorial trait with several involved genes; each one with low penetrance, so host genome background as well as other environmental factors could play a role: viral genotype is an important variable to be considered. Viral type was not available to us, since HIV-1 genotyping is performed in Brazil during

antiretroviral therapy virological failure or in specific cases. Nevertheless, HIV-1 subtype B is the most prevalent in Pernambuco state (Northeastern, Brazil), with prevalence ranging from 57% to 78% of all HIV-1 infections [36, 37].

## CONCLUSION

With these restraints in mind, our study observed an association between *HLA-C* rs9264942 SNP (C allele) and lower HIV-1 pre-treatment pVL, when compared to other genotypes, in an admixed Brazilian population, replicating several previous findings from both GWAS and case-control studies, thus corroborating the important role of HLA-C in the control of viral replication.

## ETHICS APPROVAL AND CONSENT TO PARTICIPATE

Not applicable.

## HUMAN AND ANIMAL RIGHTS

No Animals/Humans were used for studies that are base of this research.

## CONSENT FOR PUBLICATION

Not applicable.

## CONFLICT OF INTEREST

The authors declare no conflict of interest, financial or otherwise.

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