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Functional evaluation of immunoregulatory molecules HLA-G, galectin-1, and IL-10 in people living with HIV

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Abstract

Objective(s): Investigate polymorphisms and expressions of human leukocyte antigen-G (HLA-G), galectin-1 (Gal-1), and interleukin-10 (IL-10) in people living with HIV (PLHIV) with and without comorbidities to help understanding the mechanisms involved in triggering these disorders in PLHIV and in their prognosis.

Design: Here we evaluated the potential correlation between the genetic polymorphism and/or protein levels of HLA-G, Gal-1, and IL-10 with and without comorbidities of PLHIV.

Methods: Two hundred HIV patients under antiretroviral treatment (83 with comorbidities and 117 without comorbidities) and 200 healthy individuals (controls) were genotyped, using PCR, for HLA-G 14-base pair polymorphism located at the 3' untranslated region in exon 8 insertion/insertion (Ins/Ins: low HLA-G expression) or deletion/deletion (Del/Del: high HLA-G expression). Soluble levels of HLA-G (sHLA-G), Gal-1, and IL-10 were quantified by enzyme-linked immunosorbet assay.

Results: HIV patients without comorbidities exhibited higher frequency of 14-base pair Del/Del genotype than HIV patients with comorbidities. As expected, HIV patients Ins/Ins with and without comorbidities produced less sHLA-G than controls. However, HIV patients Del/Del with comorbidities expressed sHLA-G more than controls and HIV patients Del/Del without comorbidities. Interestingly, patients that showed low levels sHLA-G, and presence of comorbidities, exhibited high Gal-1 serum levels. However, an increase in soluble levels of IL-10 in PLHIV was observed when compared to controls, especially in the PLHIV group without comorbidities suggesting, a protective role of IL-10 in the development of comorbidities.

Conclusions: These data suggested that the high expression of sHLA-G and IL-10 or Gal-1 could be associated and could be associated with the development or not of comorbidities in PLHIV.

Abbreviations: 3'UTR = 3' untranslated region, ART = antiretroviral therapy, bp = base pair, CONT = healthy controls, CI = confidence interval, Del = deletion, ELISA = enzyme-linked immunosorbet assay, Gal-1 = galectin-1, HLA-G = human leukocyte antigen-G, IL-10 = interleukin-10, Ins = insertion, OR = odds ratio, PLHIV = people living with HIV, sHLA-G = soluble levels of HLA-G.

Keywords: comorbidities, galectin-1, HIV, human leukocyte antigen-G, interleukin-10, polymorphisms

1. Introduction

Early diagnosis and treatment effectiveness have made HIV infection a chronic disease.[1] HIV triggers inflammatory changes whose mechanisms have been compared with those of inflammation activated by aging, increasing the risk for age-related diseases and mortality.[2–6] In addition to the adverse effects of antiretroviral therapy (ART), these conditions promote noninfectious comorbidities, typical of the elderly, in people living with

Editor: Maya Saranathan.

This work was supported by Federal University of Espírito Santo, FAPES Research Agency (#139/2019).

The authors have no conflicts of interest to disclose.

The datasets generated during and/or analyzed during the current study are available from the corresponding author on reasonable request.

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How to cite this article: Cortelette NA, Souza NDO, Cataldi-Rodrigues L, Arthur C, Stowell SR, Dias-Baruffi M, Guimarães DAM, Ayres LR, Trés Pancoto JA. Functional evaluation of immunoregulatory molecules HLA-G, galectin-1, and IL-10 in people living with HIV. Medicine 2022;101:2(e28489).

Received: 30 July 2021 / Received in final form: 15 November 2021 / Accepted: 16 December 2021

http://dx.doi.org/10.1097/MD.0000000000028489

HIV (PLHIV) relatively younger, such as neurocognitive, cardiovascular, metabolic disorders, associated with the bone system and cancers not associated with HIV.[7]

In isolation, these disorders are related to several cytokine gene polymorphisms that alter their expressions in the clinical course of diseases.[8–10] Since HIV infection modifies the inflammatory process contributing to the development of comorbidities in ways that are still poorly understood, the analysis of gene polymorphisms of immunomodulatory molecules, as well as their expressions in PLHIV, become necessary.

Human leukocyte antigen-G (HLA-G), galectin (Gal)-1 and the cytokine interleukin (IL)-10 are described as relevant anti-inflammatory molecules in various pathological conditions.[11–14] Investigating their polymorphisms and expressions in PLHIV with and without comorbidities can help in understanding the mechanisms involved in triggering these disorders in PLHIV and in their prognosis.

HLA-G is a nonclassical HLA class I antigen that differs from classical class I molecules by its restricted tissue distribution, diversity of protein isoforms and limited gene polymorphism.[15,16] HLA-G molecule is associated with the induction of inhibitory stimuli for T and B lymphocytes, natural killer cells, and antigen-presenting cells.[17–19] The 14 base pair (bp) insertion/deletion (Ins/Del) polymorphic site in the 3' untranslated region (3'UTR) region influences HLA-G expression.[11,20] For HIV-1, the polymorphism of this region has been associated with mother-to-child fetal transmission.[21–23] The elevated expression of the soluble forms of HLA-G (sHLA-G) in HIV-1 infected people was related to the progression of HIV pathogenesis through the induction of tolerance.[24] In addition, progressive infections in PLHIV without ART have been associated with high levels of circulating sHLA-G secreted in part by monocytes and dendritic cells for autocrine regulation of their functions.[25]

Gal-1 belongs to the "Glycan-binding proteins", which specifically bind to glycans.[13,26] In the immune system, it is synthesized and secreted by a variety of cells, including activated T and B lymphocytes, macrophages, FOXP3 + Treg, tolerogenic dendritic cells, γ/δ T lymphocytes, microglia, and myelocytic suppressor cells.[27–33] The immunoregulation of Gal-1 has been associated with beneficial effects in the resolution of autoimmunity and allergies, tolerance at the maternal–fetal interface and with detrimental effects in favoring the immune escape of tumor cells and the compromise of effective antimicrobial responses.[34] In relation to HIV-1, it is proposed that there is a contribution to increase the infectious capacity of the virus by accelerating the process of binding and adhesion of the virus to the target cells.[35]

IL-10, a cytokine that suppresses the immune response, is secreted by dendritic cells (CDs), B cells, macrophages, CD4 + T cells, CD8 + T cells, and Tregs.[36,37] Its functions are related to immunoregulation, since it suppresses adaptive and innate immunity, by reducing the proliferation of T lymphocytes.[38–40] This cytokine has a dubious role, since it is extremely important in cases of exacerbated inflammatory reactions.[41–43] In contrast, this suppression of immune responses promoted by high levels of IL-10 has been associated with increased susceptibility to infectious diseases and the development of pathologies.[14,44–46] In HIV infection, immunological suppression is important for the non-exacerbation of the inflammatory response, however it increases susceptibility to other infectious diseases, in addition to facilitating the escape of the virus from the action of the immune system, a fact resulting from the induction of the production of IL-10 for HIV viral proteins, including Tat, Nef, and gp120.[14,44,46–50]

The aim of this study is to assess the potential correlation between genetic polymorphism and/or HLA-G, Gal-1, and IL-10 protein levels with and without comorbidities of PLHIV.

Investigations of the immunomodulatory mechanisms of these molecules in PLHIV may be important in the establishment of new biomarkers for diagnosis and prognosis, new therapeutic targets, for prevention of transmission, in addition to the development of immunotherapies and outcomes to cure the infection.[51,52]

2. Methods

2.1. Patients and controls

The study was performed with 200 PLHIV, without another infectious disease, (mean age 42.6 ± 12.99), 35.5% women and 64.5% men, average infection time 9 years. The patients evaluated were attended Testing and Counseling Center of Sexually transmitted infections of Vitória – Espírito Santo, 117 patients did not developed comorbidities (PLHIV-NC) and 83 patients develop comorbidities (PLHIV-DC). Healthy controls (CONT) was 200 individuals blood donors from hemocenter of Vitória – Espírito Santo, were selected from a sample consisting with case-control samples matched for age, sex, skin color in order to generate 2 homogeneous groups. The local Ethics Committee approved the protocol of the study (#2.033.231/2016) and all patients or their guardians gave written informed consent to participate.

2.2. DNA extraction

Ten milliliters of peripheral venous blood from each individual into vacutainer tubes (Becton Dickinson, Plymouth, England), containing EDTA K3 (0.055 mL/tube) for DNA extraction, using a salting out procedure adapted.[53]

2.3. HLA-G 14-bp Ins/Del polymorphism genotyping

The variability of the HLA-G 3'UTR was evaluated as previously described.[54] Briefly, DNA was amplified using the HLAG8R (5'-GTCTTCCATTTATTTTGTCTCT-3') and HLAG8F (5'-TGTGAAACAGCTGCCCTGTGT-3') primers. The amplification reaction was performed in a final volume of 25 μL, containing 10× amplification buffer (15 mM $MgCl_2$, 500 mM KCl, 100 mM Tris HCl, pH 8,4, 1% de TritonX-100), 10 mM of each dNTP, 10 pmol/μL of each primer, 50 mM $MgCl_2$, 1 U/μL of Platinum DNA polymerase (Invitrogen, Carlsbad, CA), and 80 ng/μL of genomic DNA. Cycling conditions included an initial step at 94°C for 4 minutes, followed by 34 cycles at 94°C for 45 seconds, 60°C for 40 seconds, 72°C for 45 seconds, and a final extension step at 72°C for 7 minutes. The PCR products were analyzed according to the fragment sizes (310/324 bp) by the presence or absence of a specific band in a 10% polyacrylamide gel.

2.4. Dosages of soluble forms of HLA-G (HLA-G1 and HLA-G5) and GAL-1

The measurements of the soluble forms of HLA-G (HLA-G1 and HLA-G5) were performed using the commercial kit EXBIO (Praha as, Vestec, Czech Republic) and Gal-1 using the commercial kit R&D Systems (Inc., Minneapolis, MN). The 2 kits employ the use of an enzyme-linked immunosorbet assay (ELISA) sandwich immunoassay. The dosing procedures followed the manufacturers' determinations according to their respective manuals.

Plasma samples with EDTA anticoagulant from patients and controls were selected and pooled in a sample pool according to the genotype of the HLA-G gene polymorphism and similar clinical data (age, sex, time of infection, viral load, treatment, comorbidities).

In a 96-well plate specific for ELISA tests, 81 PLHIV under ART and 4 without ART were dosed divided into groups: 13 PLHIV-DC Del/Del genotype (4 sample pool with 3 patients in each pool/well and 1 patient sample without ART/well); 15 PLHIV-NC Del/Del (5 sample pool with 3 patients in each pool/well); 13 PLHIV-DC Ins/Ins (4 sample pools with 3 patients in each pool/well and 1 patient sample without ART/well); 15 PLHIV-NC Ins/Ins (5 sample pool with 3 patients in each pool/well); 16 PLHIV-DC Ins/Del (5 sample pool with 3 patients in each pool/well and 1 patient sample without ART/well); 13 PLHIV-NC Ins/Del (4 sample pool with 3 patients in each pool/well and 1 patient sample without ART/well).

On the same plate, 27 CONT samples were divided into the following groups: 9 CONT Del/Del genotype (3 sample pool with 3 controls each pool/well); 9 CONT Ins/Ins genotype (3 sample pool with 3 controls each pool/well); and 9 CONT Ins/Del genotype (3 sample pool with 3 controls each pool/well). To calculate the results, a standard curve with white was used in each kit according to their specifications.

2.5. Dosages of soluble doses of cytokine IL-10

The measurement of the soluble level of the cytokins IL-10 were performed using Kit Human IL-10 standard Sandwich ABTS ELISA (Peprotech, Rocky Hill, NJ), respectively. All the test in question use the sandwich ELISA immunoassay method. After the development of the color, the optical density readings were performed in a spectrophotometer model 150922B EPOCH2TC BioTek Instruments in the wavelengths of 400 and 650 nm, kinetic mode, for the time recommended in the protocol developed according to the manufacturer's recommendations.

2.6. Statistical analysis

Alleles and genotypes frequencies were compared between patients and controls using the Fisher exact test, and the odds ratio (OR) and 95% confidence interval (95% CI) were estimated and was performed using Graph Pad Instat 3.05 for windows (La Jolla, CA). Considering that the 9 polymorphic sites observed at the HLA-G 3'UTR are included in a very small gene segment and considering that significant linkage disequilibria among pairs of these polymorphic was observed in the Brazilian and in several worldwide populations.[55–57] When comparing the statistical significance between the groups in the HLA-G, Gal-1, and IL-10 measurements, GraphPad Prisma software (version 1.5, San Diego, CA) was used. ANOVA ONE WAY tests were chosen and BONFERRONI and TUKEY post-tests in the HLA-G and Gal-1 measurements. Mann–Whitney and Kruskal–Wallis tests were chosen and Dunns post-tests in the IL-10 measurements. Values of P were considered statistically different when those values were $<.05$.

3. Results

3.1. Profile of comorbidities

Of the 83 PLHIV-DC, 37 (44.6%) developed metabolic disorders (dyslipidemia, type 2 diabetes mellitus, and/or chronic anemia); 30 (36.1%) neurocognitive disorders (peripheral neuropathies, depression, anxiety, insomnia, epilepsy, and/or psychiatric disorders), 29 (34.9%) cardiovascular disorders (systemic arterial hypertension and/or congestive heart failure); 11 (13.3%) developed cancers (skin cancer, larynx, cervical, ovary, thyroid, rectum, and/or Kaposi sarcoma); 11 (13.3%) autoimmune/inflammatory disorders (systemic lupus erythematosus, hypothyroidism, psoriasis, arthritis, arthrosis, gout, and/or Crohn disease); 5 (6.0%) bone disorder (osteopenia); 4 (4.8%) kidney disorders (impaired kidney function and/or chronic kidney disease); and 2 (2.4%) liver disorders (liver dysfunction). The same patient may have been categorized into different disorders according to the comorbidities developed.

Table 1

Distribution of allele and genotype frequencies (%) of 14 bp Ins/Del polymorphism in the 3'UTR region of the HLA-G gene in PLHIV-DC (n = 83) and CONT (n = 200).

14pb Ins/Del	PVHIV-DC, n (%)	CONT, n (%)	Fisher exact test
Alleles			
Ins	81 (48,80)	168 (42,00)	$P=.1629$
Del	85 (51,20)	232 (58,00)	$P=.1629$
Genotypes			
Ins/Ins	16 (19,30)	36 (18,00)	$P=.8663$
Ins/Del	49 (59,00)	96 (48,00)	$P=.1168$
Del/Del	18 (21,70)	68 (34,00)	$P<.0469$*

(%) = allele and genotype frequency, 3'UTR = 3' untranslated region, bp = base pair, Del = deletion, HLA-G = human leukocyte antigen-G, Ins = insertion, n = number of alleles and genotypes, $P=P$ value.

3.2. 14 bp Ins/Del polymorphism of the HLA-G gene

In the analysis of the 14 bp Ins/Del polymorphism of the HLA-G gene, the Del/Del genotype was less frequent in PLHIV-DC ($P<.0469$; OR = 0.5370; 95% CI = 0.2954–0.9781) when compared to CONT (Table 1). The Ins/Del genotype was more frequent in PLHIV-DC ($P<.0457$; OR = 1.8010; 95% CI = 1.0190–3.1840) when compared to PLHIV-NC. The Del/Del genotype was more frequent in PLHIC-NC when compared with PLHIV-DC ($P<.0136$; OR = 0.4431; 95% CI = 0.2333–0.8415) (Table 2).

3.3. Dosages of the soluble forms of HLA-G (HLA-G1 and HLA-G5)

The PLHIV-DC and PLHIV-NC groups of the Ins/Ins genotype showed statistically significant lower levels in relation to the

Table 2

Distribution of allele and genotype frequencies (%) of 14 bp Ins/Del polymorphism in the 3'UTR region of the HLA-G gene in PVHIV-DC (n = 83) and PVHIV-NC (n = 117).

14pb Ins/Del	PVHIV-DC, n (%)	PVHIV-NC, n (%)	Teste exato de Fisher
Alleles			
Ins	81 (48,80)	92 (39,3)	$P=.0656$
Del	85 (51,20)	142 (60,7)	$P=.0656$
Genotypes			
Ins/Ins	16 (19,30)	20 (17,1)	$P=.7120$
Ins/Del	49 (59,00)	52 (44,4)	$P<.0457$*
Del/Del	18 (21,70)	45 (38,5)	$P<.0136$*

(%) = allele and genotype frequency, 3'UTR = 3' untranslated region, bp = base pair, Del = deletion, HLA-G = human leukocyte antigen-G, Ins = insertion, n = number of alleles and genotypes, $P=P$ value.

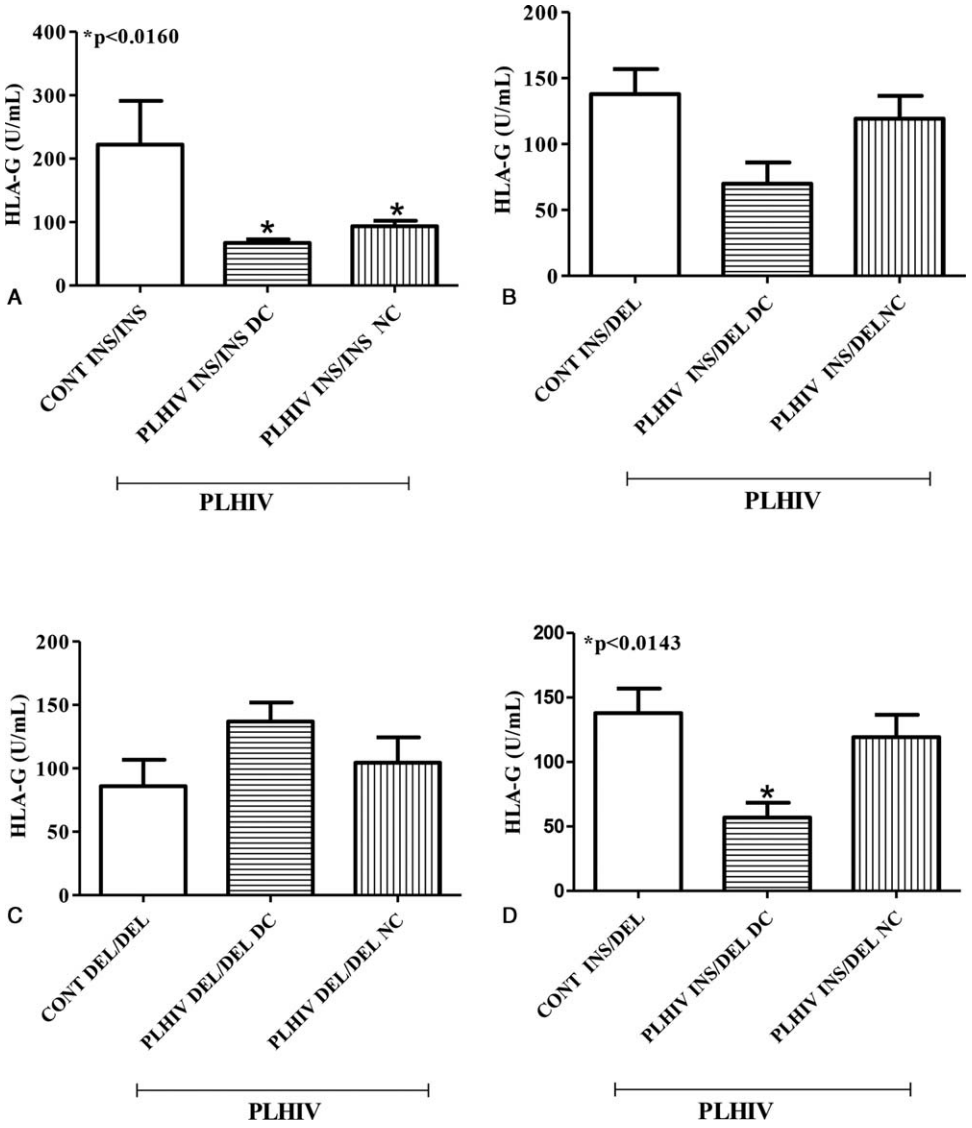

Figure 1. Quantification of soluble HLA-G by ELISA in PLHIV and CONT separated by genotypes. (A): Ins/Ins genotype; (B): Ins/Del genotype; (C): Del/Del genotype; (D): Ins/Del genotype excluding PLHIV without ART. Groups: (A): CONT Ins/Ins (n = 9), PLHIV Ins/Ins DC (n = 13), and PLHIV Ins/Ins NC (n = 15). (B): CONT Ins/Del (n = 9), PLHIV Ins/Del DC (n = 16), and PLHIV Ins/Del NC (n = 13). (C): CONT Del/Del (n = 9), PLHIV Del/Del DC (n = 13), and PLHIV Del/Del NC (n = 15). (D): CONT Ins/Del (n = 9), PLHIV Ins/Del DC, excluding those without ART (n = 15) and PLHIV Ins/Del NC, excluding those without ART (n = 12). ART = antiretroviral therapy, CONT = healthy controls, Del = deletion, ELISA = enzyme-linked immunosorbet assay, HLA-G = human leukocyte antigen-G, Ins = insertion, PLHIV = people living with HIV.

CONT Ins/Ins ($P < .0160$) (Fig. 1A). In addition, comparative analyses were carried out between the PLHIV-DC, PLHIV-NC, and CONT groups of the genotype Ins/Del (Fig. 1B), and the groups PVHIV-CC, PLHIV-NC, and CONT of the genotype Del/Del (Fig. 1C), however, no statistically significant changes were found among all groups evaluated. However, when comparing CONT, PLHIV-DC, and PLHIV-NC Ins/Del, excluding the dosages of patients without ART from the analysis, PLHIV-DC showed lower levels of soluble HLA-G compared to CONT and PLHIV-NC ($P < .0143$) (Fig. 1D).

3.4. Analysis of soluble levels of Gal-1

The PLHIV-DC group of the Ins/Ins genotype showed statistically significant higher levels compared to the PLHIV-NC Ins/Ins and CONT Ins/Ins groups ($P < .0019$) (Fig. 2A). In addition, comparative analyses were carried out between the PLHIV-DC, PLHIV-NC and CONT gen groups of Ins/Del (Fig. 2B), and between the PLHIV-DC, PLHIV-NC and CONT groups of Del/Del genotype (Fig. 2C). However, no statistically significant changes were found.

3.5. Analysis of soluble levels of IL-10

All samples were evaluated for the soluble dosage of the cytokine IL-10. The analyzes of plasma levels of the CONT, PLHIV, PLHIV-NC, and PLHIV-DC groups are presented in relation to the median and the minimum and maximum values, as shown in Table 3. A greater production of IL-10 can be observed ($P < .0001$) in the PLHIV group compared to the CONT group.

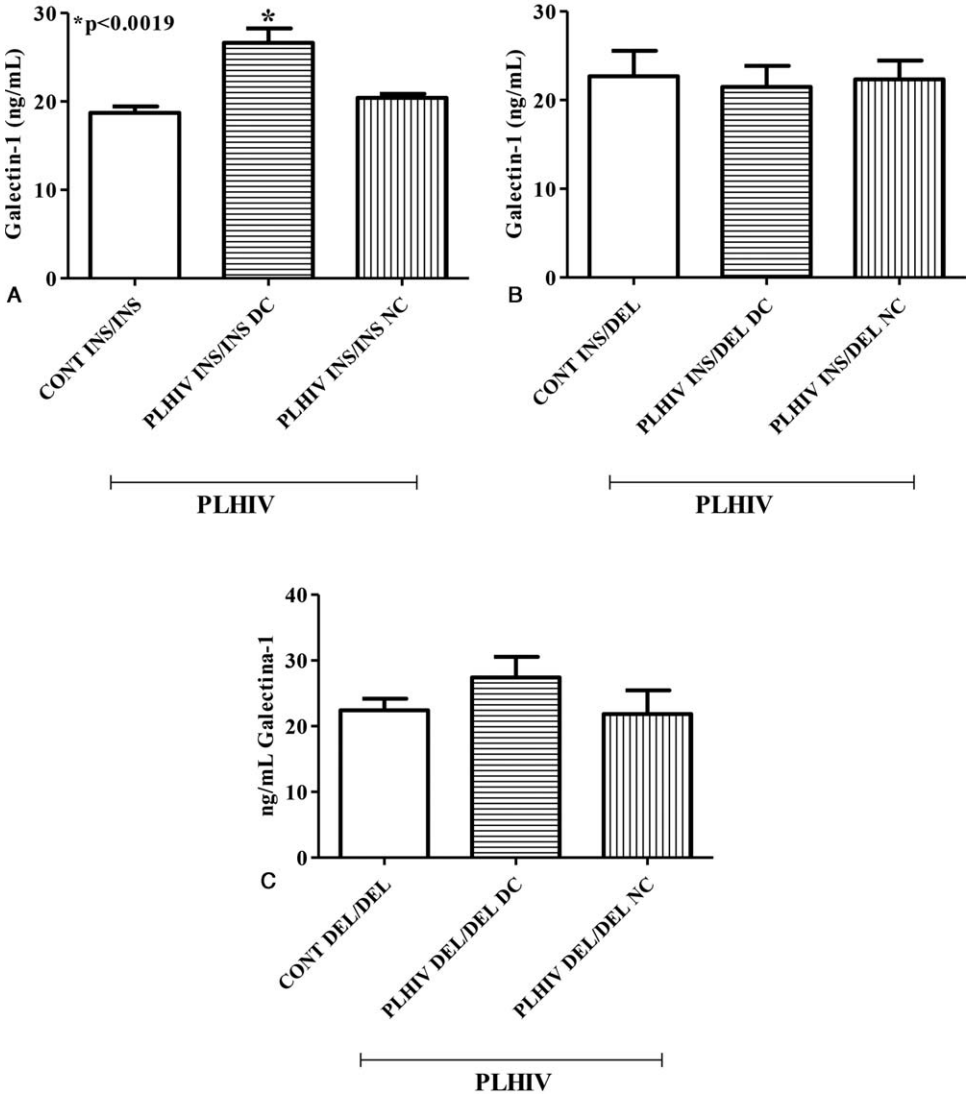

Figure 2. Quantification of soluble Gal-1 by ELISA in PLHIV and CONT classified by genotypes. Ins/Ins (A) genotype; Ins/Del (B) genotype and Del/Del (C) genotype. Groups: (A): CONT Ins/Ins (n = 9), PLHIV Ins/Ins DC (n = 13), and PLHIV Ins/Ins NC (n = 15); (B): CONT Ins/Del (n = 9), PLHIV Ins/Del DC (n = 16), and PLHIV Ins/Del NC (n = 13). (C): CONT Del/Del (n = 9), PLHIV Del/Del DC (n = 13), and PLHIV Del/Del NC (n = 15). CONT = healthy controls, Del = deletion, ELISA = enzyme-linked immunosorbet assay, Gal-1 = galectin-1, Ins = insertion, PLHIV = people living with HIV.

When comparing the PVHIV-NC and PVHIV-DC groups, a greater production is observed in the PVHIV-NC group, however without statistical significance.

Table 3

Levels of IL-10 in pg/mL, presented as median (minimum–maximum) for the CONT, PLHIV, PLHIV-NC, and PVHV-DC groups.

CONT (n = 200)	PLHIV (n = 200)	*P*
220.3 (123.3–1373)	402.5 (75.33–3485)	<.0001*
PLHIV-NC (n = 117)	**PLHIV-DC (n = 83)**	***P***
593.1 (159.2–3000)	353.4 (75.33–3485)	.1636

CONT = healthy controls, n = total number; median and minimum–maximum values, PLHIV = people living with HIV.

* $P < .05$ was considered significant.

4. Discussion

The HLA-G 14 bp Del/Del genotype is related to greater production of the molecule.[58–60] The increase or decrease in HLA-G expression may be related to the affinity of miRNAs for the 14 bp Ins/Del polymorphic region between the +2961 and +2974 position in the 3'UTR region of the HLA-G. According to data obtained in the literature, through in silico study, that the final consequence of miRNA action on the production of HLA-G mRNA by the presence/allele insertion of 14 bp would be more rigorous than the production of mRNA by the absence/14 bp deletion, which could explain the decrease in HLA-G production at 14 bp insertion.[61] In our study, the Del/Del genotype was more frequent in CONT and in PLHIV-NC than in PLHIV-DC, indicating a possible protective role of this molecule in susceptibility to HIV infection and in the development of comorbidities, since it acts by regulating processes inflammatory cells in the body.

PLHIV under ART had their sHLA-G levels decreased after starting treatment.[62] Once the viral replication is controlled by ART, the inflammatory processes decrease and, consequently, the need to produce anti-inflammatory molecules, such as HLA-G, that regulate these processes. Accordingly, progressive HIV infections in a patient without ART were associated with high levels of circulating soluble HLA-G secreted in part by monocytes and dendritic cells for autocrine regulation of their functions.[25]

In the analysis of the CONT and PLHIV groups, of the Ins/Ins genotype, less sHLA-G production was observed in both PLHIV-DC and PLHIV-NC when compared to the CONT group. The Ins/Ins genotype is associated with lower HLA-G production. It is suspected that this expression is further reduced in PLHIV, as they are on ART that controls viral replication and the inflammatory process resulting from the infection, thus reducing the need for HLA-G production to regulate inflammation.

This trend was observed in the analyses of the CONT and PLHIV groups of the Ins/Del genotype. This genotype, in our study, was more frequent in PLHIV-NC, indicating a possible greater protection from inflammatory processes, even when there is the presence of the Ins allele, whose Ins/Ins genotype has been associated with low HLA-G production.

In the comparison between the CONT and PLHIV groups with the Ins/Del genotype, when excluding the individual from the PLHIV-DC Ins/Del group who was not on ART, there was a significant decrease in the production of sHLA-G when compared with the CONT and PLHIV-NC. This was expected since HIV-infected patients on ART have a profile of decreased sHLA-G production after the introduction of therapy. This significant negative regulation of sHLA-G by ART in PLHIV-DC may indicate a contribution to the development of other comorbidities whose inflammatory mechanisms no longer rely on this important HLA-G regulation pathway.

In the evaluation of Gal-1 production, a greater significant production of this molecule was observed in the group of PLHIV-DC Ins/Ins when compared to CONT and PLHIV-NC of the same genotype. This may indicate activation of other inflammation regulating pathways, such as Gal-1, in those people with less production of sHLA-G and with other inflammatory processes underway due to comorbidities.

HIV infection causes changes in the immune system that cause increased secretion of IL-10,[63–65] as observed in the soluble dosage of the population of study, in which there was an increase in the concentration of this cytokine in all groups, PLHIV, PLHIV-NC, and PLHIV-DC, when compared to healthy individuals, CONT group.

In contrast, PLHIV-NC and PLHIV-DC groups when compared to the CONT group, thus suggesting that low levels of IL-10 may increase susceptibility to HIV-1 infection.

Thus, the expression of the IL-10 gene has a certain flexibility and plasticity which allows, through the action of antigens or even a specific cytokine environment, as occurs in HIV infection, a conformation of gene expression.

Consequently, it is suggested that the increase in plasma levels of IL-10 in the PLHIV organism may act as a protective factor against disease progression,[41,66–68] in such a way that there is a greater expression of IL-10 in those individuals where the course of the infection tends to progress more slowly. In the study scenario, it is then expected that the PLHIV-NC group will present increased concentrations of this cytokine when compared to the PLHIV-DC group, exactly as observed.

Regarding the levels of IL-10 observed in the PLHIV groups when compared to the CONT group, especially in the PLHIV-NC group, thus suggesting a protective role for IL-10 through its physiological effects on the immune system in the development of comorbidities. Moreover, the HLA-G molecule may be related to the protection of the development of comorbidities in HIV patients. Together, considering the results found in the present study, it is observed that high expression of sHLA-G and IL-10 or Gal-1 can be associated with better or worse clinical outcome in PLHIV under ART, respectively.

Acknowledgments

This study was developed at the Municipal Health Secretary of the city of Vitória – Espírito Santo and LABIOM – UFES.

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