ANALYSIS OF *Leishmania* MIMETIC NEOGLYCOPROTEINS FOR THE CUTANEOUS LEISHMANIASIS DIAGNOSIS

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KEY FINDINGS

- The disaccharide Galα1-3Galβ is the immunodominant saccharide in *Leishmania* cell surface and is the unique non-reducing terminal glycosphingolipids structure recognized by anti-α-Gal.
- Sensitivity and specificity of all NGPs ranged from 62,2% to 78,4% and 58.3% to 96,7%, respectively.
- The NGPs can be used for cutaneous leishmaniais diagnosis.

SUMMARY

Oligosaccharides are broadly present on *Leishmania* cell surfaces. They can be useful for the leishmaniases diagnosis and also helpful in identifying new cell markers for the disease. The disaccharide Galα1-3Galβ is the immunodominant saccharide in *Leishmania* cell surface and is the unique non-reducing terminal glycosphingolipids structure recognized by anti-α-Gal. This study describes an enzyme-linked immunosorbent assay (ELISA) used to measure serum levels of anti-α-galactosyl (α-Gal) antibodies in patients with cutaneous leishmaniasis (CL). Optimal ELISA conditions were established and two neoglycoproteins (NGP) containing the Galα1-3Gal terminal fraction (Galα1-3Galβ1-4GlcNAc-HAS and Galα1-3Gal-HAS) and one Galα1-3Gal NGP analogue (Galα1-3Galβ1-3GlcNAc-HAS) were used as antigens. Means of anti-α-Gal antibody titres of CL patients were significantly higher ($p<0.05$) than the healthy individuals for all NGPs tested. Sensitivity and specificity of all NGPs ranged from 62,2% to 78,4% and 58.3% to 96,7%, respectively. In conclusion, the NGPs can be used for CL diagnosis.

Key words: Antigen, ELISA, *L. braziliensis*, Saccharide, α-galactosyl.

INTRODUTION

The Leishmaniases complex are divided into three distinct forms of clinical presentation. The major factor that determines the development of each form of the disease is the specie of parasite associated with the host's specific immune responses (not all those infected by the parasites will develop the disease). In the most common form of the disease, cutaneous leishmaniasis (CL), the parasites remain only at the site of the sand fly bite and cause localized long-term ulceration with no systemic symptoms. In some cases, inadequate treatment of a CL lesion may lead to later development of mucocutaneous leishmaniasis (ML). The third clinical form is visceral leishmaniasis (VL) that is the most pathogenic in which parasites migrate from the inoculation site to multiply in the host's liver and spleen macrophages and bone marrow, causing immunosuppression and death if not treated (PEACOCK, 2007; CAMPOS *et al*., 2008; DAVID & CRAFT, 2009; BIFELD & CLOS, 2015; VAN DER AUWERA & DUJARDIN, 2015). The control of leishmaniases is presently a serious problem due to the high death rates involved and the economic losses resulting from morbidity. In addition, the disease is strongly linked to poverty in the tropical and subtropical areas and with ever increasing cases worldwide each year. Ninety-eight countries are officially considered endemic for leishmaniases and estimates show that more than 58.000 VL and 220.000 CL cases are notified per year. More than 90% of global VL cases occurring in six countries - India, Bangladesh, Sudan, South Sudan, Brazil and Ethiopia - and around 75% of global CL cases occurring in ten countries - Afghanistan, Algeria, Colombia, Brazil, Iran, Syria, Ethiopia, North Sudan, Costa Rica and Peru. (ALVAR *et al*., 2012; DE VRIES, REEDIJK & SCHALLIG, 2015).

Rapid methods for the effective leishmaniases diagnosis and species identification are urgently needed, either by prevention and control of leishmaniases in endemic areas or treatment of infected patients. The definitive diagnosis of all suspected *Leishmania* infections should be performed in an association of clinical symptoms, and parasitological and immunologic findings. Several diagnostic methods have been described to detect the presence of the *Leishmania* parasites, with a huge variation in diagnostic accuracy, including direct parasitological examination by microscopy, histopathology and/or parasite culture, indirect testing by serology and molecular diagnostics. The demonstration of the presence of the parasite by parasitological diagnosis is still considered the gold standard in leishmaniases diagnosis because of its high specificity. However, the sensitivity of the direct examinations is low for the diagnosis of CL and ML, with a range from 15% to 70%, whilst in case of VL the sensitivity varies depending on the tissue used, with a range from 93% to 99% for spleen, 53% to 86% for bone marrow and 53% to 65% for lymph node aspirates. Moreover, parasite culture in the culture media is

difficult, requires technical skills, is prone to contamination, and is time-consuming. As all other parasitological method for *Leishmania* detection, the parasite culture does not allow *Leishmania* species determination. The sensitivity of cultures depends on the parasite quantity, but is estimated to be between 60% and 85%. (BHARGAVA & SINGH, 2012; REZVAN, 2014; DE VRIES, REEDIJK & SCHALLIG, 2015; GEORGIADOU, MAKARITSIS & DALEKOS, 2015; PAIVA-CAVALCANTI *et al.*, 2015).

Alternative and complementary diagnosis techniques also include the evaluation of indirect parameters. The Montenegro skin test has been successfully used in the diagnosis of cutaneous and mucocutaneous forms of the disease. Its sensitivity range from 86.4%, but it is not very reliable for detecting the presence of parasites in recent lesions, in diffuse forms of disease and in immunosuppressed patients, as well as not differentiating between past and present infection (GOTO & LINDOSO, 2012; DE VRIES, REEDIJK & SCHALLIG, 2015; HANDLER *et al*., 2015; PAIVA-CAVALCANTI *et al*., 2015).

Immunological diagnoses, another important indirect method for detecting leishmaniases, are based on the detection of either *Leishmania* antigens or anti-*Leishmania* antibodies in the host serum samples. The optimal test for serologic diagnosis is one that is easy to use, cheap to make and has both a high sensitivity and specificity. Most of the immunological techniques for the detection of anti-*Leishmania* antibodies have been based on the Enzyme-Linked Immunosorbent Assay (ELISA) technique and the sensitivity and specificity of this method depends on the antigen employed. Considering the variations of the individual immune responses to the infection, several antigens have been identified for potential use in the diagnosis of leishmaniases (GOTO & LINDOSO, 2012; MAIA *et al*., 2012; SÁNCHEZ-OVEJERO *et al*., 2016).

Oligosaccharides are broadly present on cell surfaces arranged as glycan arrays, responsible for regulating the interaction between cells. They can be useful for disease diagnosis and also helpful in identifying new disease-causing microbial cell markers. The interactions among pathogens and their host cells are guided by different cell-surface glycans and glycan binding receptors at each stage of the infectious process (FERNÁNDEZ-TEJADA *et al*., 2015). Glycoinositol phospholipids (GIPLs) are the main family of low molecular weight glycolipids synthesized by *Leishmania* parasites. It is expressed in abundance on promastigote and amastigote cell surfaces and is not linked to proteins, forming a protective surface revetment that provides essential host-parasite interactions. However, each parasite stage is already known to have a different glycoconjugate attached on its surface beyond GIPLs. Biochemical analyses reveal that, unlike amastigotes, the procyclic promastigotes surface coat expresses two other glycoconjugates which are less abundant than GIPLs and are responsible for protecting parasites from hydrolytic enzymes in the sandfly gut: 1)

glycosylphosphatidylinositol (GPI) anchored to macromolecules such as metalloprotease and; 2) protein-free lipophosphoglycan complex (LPG), a GPI-anchored macromolecules underlying layer composed of densely, free-packed glycolipids. Altogether these molecules create an effective barrier which protects promastigotes from cell death processes like lysis mediated by complement system, oxygen radicals and hydrolases in the mammalian and insect host environments (ILGOUTZ *et al*., 2001; MUKHOPADHYAY *et al*., 2006; BARRETO-BERGTER *et al*., 2010; GALILI, 2013a).

High levels of antibody produced against GIPLs have been reported in people infected with trypanosomatid parasites (ÁVILA *et al*., 1988; 1989; 1991; ALMEIDA *et al*., 1994). Remarkably, anti-α-Gal recognizes specifically the unique glycosphingolipids structures known as the GPI-anchored mucins in the trypomastigote stage of *Trypanosoma* spp (GALILI, 1993; MARCHER; GALILI, 2008; SCHOCKER *et al*., 2016). However, the parasites escape through the antibodies' action by penetrating tissues and continue to produce and release GIPLs and LPG with α-Gal epitopes, stimulating the immune system to continuously produce anti-α-Gal antibodies at high titres (GALILI, 2013a). Quantities of anti-α-Gal antibodies constantly produced can also represent a continuous immune response to Galα1-3 Gal structures found in various gastrointestinal bacteria, confirming the polyreactive nature of these antibodies in human serum (GALILI, 1984; GALILI *et al.*, 1987; GALILI, 2013b).

The major GIPLs found in these parasites' cell membranes include tetraglycosylinositol, pentaglycosylinositol, and hexaglycosylphosphatidylinositol and sugar analysis of *L. mexicana* and *L. braziliensis* GIPLs revealed monosaccharide composition of Manose (Man), Galactose (Gal), Glucosamine (GlcN), and inositol (ÁVILA *et al*., 1991; MCCONVILLE & FERGUSON, 1993).

Anti-α-Gal is the most abundant natural circulating human antibody and, since it binds specifically to the Galα1–3Gal glycosphingolipids (GALILI, 1993; MARCHER *et al*., 2008; SCHOCKER *et al.*, 2016), it is assumed that the majority of the anti-α-Gal binding sites expressed in the cell membranes of many organisms have this non-reducing terminal structure (GALILI *et al*., 1988; GALILI, 1993; GALILI *et al*., 1995; GALILI, 2013a, b). However, quantities of anti-α-Gal antibodies constantly produced can also represent a continuous immune response to Galα1-3 Gal structures found in various gastrointestinal bacteria (GALILI, 1984; GALILI *et al*., 1987; GALILI, 2013b), confirming the polyreactive nature of these antibodies in human serum (SATAPATHY *et al*., 1999).

The α-Gal has the potential for many clinical uses once anti-Gal antibodies are widely produced in healthy humans and α-gal epitopes can be easily synthesized by several methods. This study describes an

standardized enzyme-linked immunosorbent assay (ELISA) used to measure serum levels of anti-α-Gal antibodies in individuals with cutaneous leishmaniasis (CL) from different endemic regions in Brazil and compares them to those from healthy individuals living in the same endemic areas and non-endemic areas using different neoglycoproteins (NGPs) from the antigen.

MATERIALS AND METHODS

Sample collection

A total of 149 serum samples from females and males of different age groups were collected from CL endemic and non-endemic regions in Brazil. The main inclusion criterion was the proven diagnosis of CL by parasite presence on the direct examination of lesion smears obtained from the edge of the active lesion with a punch biopsy tool and/or positivity in the parasite culturing. The samples were divided into seven groups (Table 1): 1) H1 (23): healthy individuals from non-endemic areas; 2) H2 (37): healthy individuals from endemic areas; 3) CL1 (12): CL patients with active lesion and no treatment; 4) CL2 (5): CL patients with active lesion under the treatment; 5) CL3 (13): CL patients that had finished treatment and were under observation for the subsequent 3 months; 6) OD (54): serum reagent patients for other diseases such as Hepatitis B and C, Syphilis and truly positive patients for Tuberculosis; 7) CD (5): serum reagent patients for Chagas disease.

Individuals from H2 group were medically examined to discard any previous CL infection. CL patients were treated according to the Brazilian Healthy Ministry guideline, with meglumine antimoniate and amphotericin B. Patients with other diseases such as Chagas disease were also studied to evaluate the chance of cross-reactivity in these tests.

Ethical approval was obtained from the Universidade Federal do Paraná Ethical Committee under number 684.244.

Enzyme-linked immunosorbent assay (ELISA)

To determine whether levels of anti-α-Gal antibodies in human serum, both non-infected and infected with CL and other diseases can identify *Leishmania* α-Gal epitopes, optimal ELISA conditions were established, such as dilutions of sera (1:100, 1:200, 1:400 and 1:800), conjugate (1:5,000, 1:10,000 and 1:20,000) and

antigens (0.1 , 0.5 and 1 µg/well). Once the best condition was established, polystyrene microtiter plates with 96 wells (NUNC C96 Immuno Plate Maxisorp Surface, Thermo Scientific) were coated overnight at 4°C with 100 µL of 0,1 µg/well of two Galα1-3Gal neoglycoprotein series with 3-atom spacer (Galα1-3Galβ1-4GlcNAc conjugated with human albumin serum , identified as NGP 2334; and Galα1-3Gal conjugated with human albumin serum, identified as NGP2203 - Dextra Laboratories) and one Galα1-3Gal analogue neoglycoprotein with 3-atom spacer (Galα1-3Galβ1-3GlcNAc conjugated with human albumin serum, identified as NGP2333 - Dextra Laboratories), diluted in carbonate–bicarbonate buffer (pH 9.6). In addition, the β-Gal NGP β1-4-Galactosyl-Galactose conjugated with bovine serum albumin and with 3-atom spacer (NGP 0204, Dextra Laboratories) was also included in the study as the "β-control" and soluble proteins from the crude extract from *Leishmania (Viannia) braziliensis* culture (strain MHOM/BR/84/LTB300) as a positive control. Human albumin serum was chosen for the generation of NGPs because of its absence of immunological reaction with human serum and its adequacy as a carrier protein.

The following day, the plates were washed twice with 200 µL/well of washing solution (NaCl 0.9% + Tween 20 at 0.05%), then the wells were blocked with 120 µL of blocking solution (Pierce Protein - Free T20 – PBS Blocking Buffer, Thermo Scientific) for one hour at 37°C, and after were washed twice again with 200 µL/well of washing solution. Immediately after, serum samples were diluted to 1:800 in a solution of PBS – 0.25% casein + 0.5% Tween 20 and were added to their respective wells and incubated at 37 °C for one hour. Then the plates were washed four times with 200 µL/well of washing solution and polyclonal rabbit anti-human IgG HRP conjugate (1.3 g/L, Dako) was diluted to 1:5,000 and was added to each well for one hour at 37°C. Finally, the reaction was developed by adding 100 µL of SureBlue TMB™ Microwell Peroxidase Substrate to each well at room temperature for 15 minutes, avoiding light, and to interrupt the reaction 20 µL of a solution 1:20 of H_2SO_4 was added. Plates were read in the Infinite F200 PRO multimode reader (Tecan) at 450 nm and values were expressed in absorbance. Each sample was measured in triplicate, including the "no serum" control for preventing the background from the secondary antibody (blank control), and the whole assay described above was performed in duplicate. The absorbance readings were performed subtracting the mean of blank control from the mean of the unknown samples in triplicate.

Determination of α-Gal specific activity

Based on Al-Salem *et al*. (2014) CBAG treatment protocol to determine the α-Gal specific activity, 0.1 μg/well of each NGP previously tested, except for NGP 2204, were treated overnight at 28°C with 0.04 U/well of α-galactosidase from green coffee beans (Sigma). After incubation, the plates were washed five times with washing solution and the ELISA was performed as described above. A pool of seven serums from CL1 and CL2 and 10 from H1 individuals was also used as positive and negative controls, respectively.

Statistical analysis

The homogeneity of variance analysis and the Kolmogorov-Smirnov test for the normal condition of variables evaluation were performed using the Statistica 7 and MedCalc 16.1 software, respectively. Once data showed non-parametric distribution, a Kruskal-Wallis one-way analysis with a Dunn post-test was performed to validate the significant difference among groups. The Mann-Whitney analysis was used to compare the means of each group between antigens and positive and negative controls. All non-parametric analyses were performed using GraphPad Prism 6 software, assuming the significant level of $p<0.05$. Finally, Receiver Operating Characteristics (ROC) curve were performed based on the logistic regression model, considering the classification of the samples (presence or absence of the disease) as a dependent variable and each antigen as an independent variable. Logistic regression model, ROC curve and sensitivity and specificity analyses were performed using R software (R CORE TEAM, Version 3.4.0, 2017) with auxiliary pROC system (Robin et al., 2011) and a significant level of $p<0.05$ was adopted.

RESULTS

Enzyme-linked immunosorbent assay (ELISA)

All serum samples collected were analysed on two occasions. First, absorbance values of samples from H1 and CL1 groups were used to perform the ROC curve analysis, sensitivity, specificity and odds ratio of all antigens. The results obtained were compared with the same parameters of the same sample groups for the positive control (Figure 1 and table 2). The cut-off values for CL were: 0,030 for NGP0204, 0,135 for *L.*

braziliensis crude extract and NGP2333, 0,131 for NGP2203 and 0,550 for NGP 2334; whilst sensitivity and specificity ranged from 62,2% (NGP 2203) to 78,4% (*L. braziliensis* crude extract), and 58.3% (NGP 0204) to 96,7% (NGP2334), respectively.

The presence of *Leishmania* anti-α-Gal antibodies was determined by comparing anti-α-Gal levels in CL patients and healthy individuals living in the same endemic area, non-endemic area and individuals with other diseases (Table 3). It was found that means of anti-α-Gal antibody titres of only CL2 and CL3 groups were significantly higher ($p<0.05$) than the H1 group for NGP 2234 and NGP 2333. For NGP 2203, all CL groups presented significantly higher means of antibody levels ($p<0.05$) compared to H1 group. No cross-reaction between the NGPs and antibodies from patients with other diseases was detected, except for the Chagas disease (CD), since the etiological agent of this disease is another trypanosomatid parasite.

When comparing each sera group for each antigen singly (Table 4), means of antibody titres of CL1 group from all NGPs showed no significant difference compared to the same group from *L. braziliensis*. However, means of antibody titres of CL2 from NGP 2334 and CL3 groups from all NGPs showed higher difference compared to the same groups *L. braziliensis*. Also, means of antibody titres of CL3 and CD groups from NGP 0204 showed lower significant differences compared to all NGPs, indicating that the β-Gal NGP is not able to be detected by antibodies of CD patients and/or CL patients after treatment.

Determination of α-Gal specific activity

To determine the specific immunogenic activity of the α-Gal residues, all NGPs, except for NGP 2204, were treated with α-galactosidase enzyme from green coffee beans, which specifically unlinks Galα1-3Gal into two parts - Galα1 and 3Gal – and, consequently, abrogates the recognition of α-Gal epitope by anti-α-Gal antibodies (Figure 2). Although it is clear there is still an antigen-antibody reaction detected in the assay performed with the presence of the α-galactosidase enzyme, compared to the controls, patients from the CL1/CL2 and CL3 groups used in enzymatic treatment assay with NGP 2334 showed a 2- and 3-fold decrease in the antibody titres than positive control, respectively. The same result was found for the other antigens: a 2.5- and 4-fold decrease in the antibody titres for CL1/CL2 and CL3 groups for NGP 2333 antigen, respectively; and 2.5- and 4-fold for the CL1/CL2 and CL3 groups NGP 2203 antigen, respectively. H2 and H1 groups used in enzymatic treatment also presented a decrease in the antibody titres when compared to the positive and negative controls from non-enzymatic treatment.

DISCUSSION

Different NGP sequences were able to be better detected by anti-α-Gal antibodies from CL patients under treatment and those that were under observation post-treatment compared to active CL patients, pointing out that the increase in the anti-α-Gal levels could be used as a potential biomarker for detection the presence of CL. However, means of antibody titres of CL patients with active lesions showed no difference compared to the same group for positive control, meaning that all NGPs tested were able to detect anti-*Leishmania* antibody levels in CL patients with active lesions at the same level of the crude extract from *L. braziliensis*. Previous studies also suggest that sera from patients with active *Leishmania* lesions and cured individuals were able to recognize α-Gal epitopes (ÁVILA *et al*., 1989; 1990; AL-SALEM *et al*., 2014).

When *Leishmania* spread through the mammalian host cells, the parasites are rarely exposed directly to the humoral immune response and the presence of antibodies is induced by the complement system when in contact with the *Leishmania* membrane (BIFELD *et al*., 2015). Levels of anti-α-Gal antibodies increase as the disease progresses (ÁVILA *et al.*, 1990), also giving support in explaining why few truly infected individuals had presented anti-α-Gal levels below the cut-off line. Either total parasite clearance can takes several years to complete or the treatment could allow a parasite cell lysis, releasing more α-Gal epitopes to the host immune system.

No cross-reaction between the NGPs and antibodies from patients with other diseases was detected, except for the Chagas disease, since the etiological agent of this disease is another trypanosomatid parasite. Ashmus *et al.* (2013) also showed that chagasic anti-α-Gal antibodies strongly recognize saccharides containing the non-reducing terminal disaccharide Galα1-3Galβ moiety.

The presence of different antibodies binding to similar epitopes, either in CL or patients with chronic *Trypanosoma* infections can indicate the strong presence of highly immunogenic oligosaccharide antigens linked to phosphatidylinositol in trypanosomatid parasites (ÁVILA *et al*., 1988; 1991). Anti-α-Gal antibodies specifically interact with glycoconjugates that have Galα1-3Galβ1-4GlcNAc residues but do not interact with Galα1-4Galβ1-4GlcNAc, β-galactosyl, or glycoconjugates with other carbohydrate residues. The α-Gal binding site has a size corresponding to the free trisaccharide Galα1-3Galβ1-4GlcNAcα- and it exhibits seven-fold higher in affinity of human anti-α-Gal than that to the disaccharide Galα1-3Gal, and much more strongly than α-Gal alone (GALILI *et al.*, 1985; GALILI, 1989; 2013b; OBUKHOVA *et al*., 2007; SCHOCKER *et al*., 2016). A decrease of antibody titres from CL patients was noticed when the α-Gal residues specific immunogenic activity

protocol was applied. The same results were observed by Galili *et al.* (1984) and Ávila *et al.* (1988; 1989; 1990; 1991), suggesting that GPIs oligosaccharide chains have only terminal α-galactose residues. It can lead to the conclusion that the disaccharide Galα1-3Galβ is the immunodominant saccharide in *Leishmania* cell surface and is the unique non-reducing terminal glycosphingolipids structure recognized by anti-α-Gal.

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Table 1. Demographic data of the study cohort. H1: healthy individuals from non-endemic areas, H2: healthy individuals from endemic areas, CL1: CL patients with active lesion and no treatment, CL2: CL patients with active lesion under the treatment, CL3: CL patients that had finished treatment and were under observation for the subsequent 3 months, OD: serum reagent patients for other diseases such as Hepatitis B and C, Syphilis (diagnosis performed by a blood bank) and truly positive patients for Tuberculosis , CD: serum reagent patients for Chagas disease (diagnosis performed by a blood bank).

Group	Sample code	Diagnosis	At the moment of the blood collection, the patient was...
Group H1	NCNE 01	healthy individuals from non-endemic area	No treatment
	NCNE 03	healthy individuals from non-endemic area	No treatment
	NCNE 04	healthy individuals from non-endemic area	No treatment
	NCNE 05	healthy individuals from non-endemic area	No treatment
	NCNE 06	healthy individuals from non-endemic area	No treatment
	NCNE 07	healthy individuals from non-endemic area	No treatment
	NCNE 08	healthy individuals from non-endemic area	No treatment
	NCNE 09	healthy individuals from non-endemic area	No treatment
	NCNE 10	healthy individuals from non-endemic area	No treatment
	NCNE 12	healthy individuals from non-endemic area	No treatment
	NCNE 13	healthy individuals from non-endemic area	No treatment
	NCNE 14	healthy individuals from non-endemic area	No treatment
	NCNE 15	healthy individuals from non-endemic area	No treatment
	NCNE 16	healthy individuals from non-endemic area	No treatment
	NCNE 17	healthy individuals from non-endemic area	No treatment
	NCNE 18	healthy individuals from non-endemic area	No treatment
	NCNE 19	healthy individuals from non-endemic area	No treatment
	NCNE 20	healthy individuals from non-endemic area	No treatment
	NCNE 21	healthy individuals from non-endemic area	No treatment
	NCNE 22	healthy individuals from non-endemic area	No treatment
	NCNE 23	healthy individuals from non-endemic area	No treatment
	NCNE 24	healthy individuals from non-endemic area	No treatment
	NCNE 25	healthy individuals from non-endemic area	No treatment
Group H2	NCE 01	healthy individuals from endemic area	Having treatment for high blood pressure and rheumatism
	NCE 02	healthy individuals from endemic area	Having treatment for high blood pressure and myalgia
	NCE 03	healthy individuals from endemic area	No treatment
	NCE 04	healthy individuals from endemic area	No treatment
	NCE 05	healthy individuals from endemic area	No treatment
	NCE 07	healthy individuals from endemic area	Having treatment for high blood pressure

Group	Sample code	Diagnosis	At the moment of the blood collection, the patient was...
Group H2	NCE 08	healthy individuals from endemic area	No treatment
	NCE 09	healthy individuals from endemic area	No treatment
	NCE 10	healthy individuals from endemic area	No treatment
	NCE 11	healthy individuals from endemic area	Having treatment for high blood pressure
	NCE 12	healthy individuals from endemic area	No treatment
	NCE 13	healthy individuals from endemic area	Having treatment for high blood pressure
	NCE 14	healthy individuals from endemic area	No treatment
	NCE 15	healthy individuals from endemic area	No treatment
	NCE 16	healthy individuals from endemic area	No treatment
	NCE 17	healthy individuals from endemic area	No treatment
	NCE 18	healthy individuals from endemic area	Having treatment for hypercholesterolemia (sinvastatine)
	NCE 19	healthy individuals from endemic area	No treatment
	NCE 20	healthy individuals from endemic area	No treatment
	NCE 21	healthy individuals from endemic area	Having treatment for high blood pressure
	NCE 22	healthy individuals from endemic area	Having treatment for rheumatism
	NCE 23	healthy individuals from endemic area	No treatment
	NCE 24	healthy individuals from endemic area	No treatment
	NCE 25	healthy individuals from endemic area	No treatment
	NCE 26	healthy individuals from endemic area	No treatment
	NCE 27	healthy individuals from endemic area	No treatment
	NCE 29	healthy individuals from endemic area	No treatment
	NCE 30	healthy individuals from endemic area	No treatment
	P02	healthy individuals from endemic area	No treatment
	P03	healthy individuals from endemic area	No treatment
	P04	healthy individuals from endemic area	No treatment
	P05	healthy individuals from endemic area	No treatment
	P09	healthy individuals from endemic area	No treatment
	P10	healthy individuals from endemic area	No treatment
	P11	healthy individuals from endemic area	No treatment
	P13	healthy individuals from endemic area	No treatment
	P14	healthy individuals from endemic area	No treatment
Group CL1	L07	Skin lesion for and positive parasitological examination by microscopy	Hadn't started the treatment yet
	L24	Skin lesion and positive parasitological examination by microscopy	Hadn't started the treatment yet
	D. K. S. M. N.	Skin lesion, positive intradermal reaction (15 mm) and positive parasite culture	Hadn't started the treatment yet
	R. L. J.	Disseminated skin lesions, positive intradermal reaction (12 mm) and positive parasite culture	Hadn't started the treatment yet
	T. R.	Disseminated skin lesions, positive intradermal reaction (21x26 mm) and positive parasite culture	Hadn't started the treatment yet
	O. G. T.	Skin lesion (1.5 cm), positive intradermal reaction (13x19 mm) and positive parasite culture	Hadn't started the treatment yet

Group	Sample code	Diagnosis	At the moment of the blood collection, the patient was...
Group CL1	W. A. B.	Skin lesion, positive intradermal reaction (12x13 mm) and positive parasite culture	Hadn't started the treatment yet
	J. V. V.	Disseminated skin lesions for 9 months and positive parasite culture	Hadn't started the treatment yet
	J. P. S.	Positive parasite culture	Hadn't started the treatment yet
	A. O. G.	Positive intradermal reaction (21x24 mm) and positive parasite culture	Hadn't started the treatment yet
	A. R. S.	Positive intradermal reaction (26x32 mm) and positive parasite culture	Hadn't started the treatment yet
	M. L. C.	Skin lesion and positive parasite culture	Hadn't started the treatment yet
	P07	Skin lesion and positive intradermal reaction	Hadn't started the treatment yet
	P16	Skin lesion and positive intradermal reaction	Hadn't started the treatment yet
Group CL2	L01	Skin lesion and positive parasitological examination by microscopy	Under treatment
	L02	Skin lesion and positive parasitological examination by microscopy	Under treatment
	L05	Skin lesion and positive parasitological examination by microscopy	Under treatment
	L08	Skin lesion and positive parasitological examination by microscopy	Under treatment
	L09	Skin lesion and positive parasitological examination by microscopy	Under treatment
	P01	Skin lesion and positive intradermal reaction	Under treatment
	P06	Skin lesion and positive intradermal reaction	Treatment had been interrupted by patient. Active lesion still remained
	P08	Skin lesion and positive intradermal reaction	Treatment had been interrupted by patient. Active lesion still remained
	P15	Skin lesion and positive intradermal reaction	Under treatment
Group CL3	L03	Skin lesion and positive parasitological examination by microscopy	Under observation during 3 months
	L04	Skin lesion and positive parasitological examination by microscopy	Under observation during 3 months
	L06	Skin lesion and positive parasitological examination by microscopy	Under observation during 3 months
	L10	Skin lesion and positive parasitological examination by microscopy	Under observation during 3 months
	L11	Skin lesion and positive parasitological examination by microscopy	Treatment had finished
	L12	Skin lesion and positive parasitological examination by microscopy	Under observation during 3 months
	L13	Skin lesion and positive parasitological examination by microscopy	Under observation during 3 months
	L15	Skin lesion and positive parasitological examination by microscopy	Under observation during 3 months
	L16	Skin lesion and positive parasitological examination by microscopy	Under observation during 3 months
	L19	Skin lesion and positive parasitological examination by microscopy	Under observation during 3 months

Group	Sample code	Diagnosis	At the moment of the blood collection, the patient was...
Group CL3	L20	Skin lesion and positive parasitological examination by microscopy	Under observation during 3 months
	L22	Skin lesion and positive parasitological examination by microscopy	Under observation during 3 months
	L23	Skin lesion and positive parasitological examination by microscopy	Under observation during 3 months
	P12	Skin lesion and positive intradermal reaction	Under observation during 3 months
Group OD	AD 02	Positive for Syphilis	No treatment
	AD 03	Positive for Syphilis	No treatment
	AD 04	Positive for Syphilis	No treatment
	AD 05	Positive for Syphilis	No treatment
	AD 06	Positive for Syphilis	No treatment
	AD 07	Positive for Syphilis	No treatment
	AD 08	Positive for Syphilis	No treatment
	AD 09	Positive for Syphilis	No treatment
	AD 10	Positive for Hepatitis B	No treatment
	AD 11	Positive for Hepatitis B	No treatment
	AD 12	Positive for Hepatitis B	No treatment
	AD 13	Positive for Hepatitis B	No treatment
	AD 14	Positive for Hepatitis B	No treatment
	AD 15	Positive for Hepatitis B	No treatment
	AD 16	Positive for Hepatitis B	No treatment
	AD 17	Positive for Hepatitis B	No treatment
	AD 18	Positive for Hepatitis C	No treatment
	AD 19	Positive for Hepatitis C	No treatment
	AD 20	Positive for Hepatitis C	No treatment
	AD 21	Positive for Hepatitis C	No treatment
	AD 22	Positive for Hepatitis C	No treatment
	AD 23	Positive for Hepatitis C	No treatment
	AD 24	Positive for Hepatitis C	No treatment
	AD 25	Positive for Hepatitis C	No treatment
	01	Positive for bacilloscopy and/or *M. tuberculosis* culture and tuberculin skin reaction ≥ 5 mm	Treatment for Tuberculosis
	02	Positive for bacilloscopy and/or *M. tuberculosis* culture and tuberculin skin reaction ≥ 5 mm	Treatment for Tuberculosis
	03	Positive for bacilloscopy and/or *M. tuberculosis* culture and tuberculin skin reaction ≥ 5 mm	Treatment for Tuberculosis
	04	Positive for bacilloscopy and/or *M. tuberculosis* culture and tuberculin skin reaction ≥ 5 mm	Treatment for Tuberculosis
	05	Positive for bacilloscopy and/or *M. tuberculosis* culture and tuberculin skin reaction ≥ 5 mm	Treatment for Tuberculosis
	06	Positive for bacilloscopy and/or *M. tuberculosis* culture and tuberculin skin reaction ≥ 5 mm	Treatment for Tuberculosis
	07	Positive for bacilloscopy and/or *M. tuberculosis* culture and tuberculin skin reaction ≥ 5 mm	Treatment for Tuberculosis

Group	Sample code	Diagnosis	At the moment of the blood collection, the patient was...
Group OD	08	Positive for bacilloscopy and/or *M. tuberculosis* culture and tuberculin skin reaction ≥ 5 mm	Treatment for Tuberculosis
	09	Positive for bacilloscopy and/or *M. tuberculosis* culture and tuberculin skin reaction ≥ 5 mm	Treatment for Tuberculosis
	10	Positive for bacilloscopy and/or *M. tuberculosis* culture and tuberculin skin reaction ≥ 5 mm	Treatment for Tuberculosis
	11	Positive for bacilloscopy and/or *M. tuberculosis* culture and tuberculin skin reaction ≥ 5 mm	Treatment for Tuberculosis
	12	Positive for bacilloscopy and/or *M. tuberculosis* culture and tuberculin skin reaction ≥ 5 mm	Treatment for Tuberculosis
	13	Positive for bacilloscopy and/or *M. tuberculosis* culture and tuberculin skin reaction ≥ 5 mm	Treatment for Tuberculosis
	14	Positive for bacilloscopy and/or *M. tuberculosis* culture and tuberculin skin reaction ≥ 5 mm	Treatment for Tuberculosis
	15	Positive for bacilloscopy and/or *M. tuberculosis* culture and tuberculin skin reaction ≥ 5 mm	Treatment for Tuberculosis
	16	Positive for bacilloscopy and/or *M. tuberculosis* culture and tuberculin skin reaction ≥ 5 mm	Treatment for Tuberculosis
	17	Positive for bacilloscopy and/or *M. tuberculosis* culture and tuberculin skin reaction ≥ 5 mm	Treatment for Tuberculosis
	18	Positive for bacilloscopy and/or *M. tuberculosis* culture and tuberculin skin reaction ≥ 5 mm	Treatment for Tuberculosis
	19	Positive for bacilloscopy and/or *M. tuberculosis* culture and tuberculin skin reaction ≥ 5 mm	Treatment for Tuberculosis
	20	Positive for bacilloscopy and/or *M. tuberculosis* culture and tuberculin skin reaction ≥ 5 mm	Treatment for Tuberculosis
	21	Positive for bacilloscopy and/or *M. tuberculosis* culture and tuberculin skin reaction ≥ 5 mm	Treatment for Tuberculosis
	22	Positive for bacilloscopy and/or *M. tuberculosis* culture and tuberculin skin reaction ≥ 5 mm	Treatment for Tuberculosis
	23	Positive for bacilloscopy and/or *M. tuberculosis* culture and tuberculin skin reaction ≥ 5 mm	Treatment for Tuberculosis
	24	Positive for bacilloscopy and/or *M. tuberculosis* culture and tuberculin skin reaction ≥ 5 mm	Treatment for Tuberculosis
	25	Positive for bacilloscopy and/or *M. tuberculosis* culture and tuberculin skin reaction ≥ 5 mm	Treatment for Tuberculosis
	26	Positive for bacilloscopy and/or *M. tuberculosis* culture and tuberculin skin reaction ≥ 5 mm	Treatment for Tuberculosis
	27	Positive for bacilloscopy and/or *M. tuberculosis* culture and tuberculin skin reaction ≥ 5 mm	Treatment for Tuberculosis
	28	Positive for bacilloscopy and/or *M. tuberculosis* culture and tuberculin skin reaction ≥ 5 mm	Treatment for Tuberculosis
	29	Positive for bacilloscopy and/or *M. tuberculosis* culture and tuberculin skin reaction ≥ 5 mm	Treatment for Tuberculosis
	30	Positive for bacilloscopy and/or *M. tuberculosis* culture and tuberculin skin reaction ≥ 5 mm	Treatment for Tuberculosis
Group CD	AD 26	Positive for Chagas disease	No treatment
	AD 27	Positive for Chagas disease	No treatment
	AD 28	Positive for Chagas disease	No treatment
	AD 29	Positive for Chagas disease	No treatment
	AD 30	Positive for Chagas disease	No treatment

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Table 2. Odds ratio for all antigens and diseases, considering H1 and CL1 groups as truly negative and positive groups, respectively. The p values with an asterisk show a significant difference between groups ($p<0.05$). NGP 2334: Galα1-3Galβ1-4GlcNAc-HAS, NGP 2333: Galα1-3Galβ1-3GlcNAc-HAS, NGP 2203: Galα1-3Gal-HAS, NGP 02040: β1-4-Galactosyl-Galactose-BSA.

	Leishmaniasis	Chagas Disease	Other Diseases
NGP 2334	77.31 (13.50-752.64)	84.84 (6.95-8263.79)	0.63 (0.08-4.55)*
NGP 2333	3483.61 (81.96-348014.7)	76385.64 (88.63-3.013784e+10)	2.17 (0.06-92.72)*
NGP 2203	1461.41 (41.97-148004.3)	133490.7 (21.39-1.771938e+12)	9.09 (0.61-342.99)*
NGP 0204	7.33 (0.24-1544.74)*	20.28 (0.00-1.148305e+04)*	0.32 (0.00-74.50)*
***L. braziliensis* crude extract**	89665263 (40916.92-3.694421e+12)	3294390465 (2847.43-1.445273e+20)	19527.46 (22.44-58990714)

Table 3. Kruskal-Wallis statistical analyses of levels of anti-α-Gal antibodies found in individuals from Cutaneous Leishmaniasis endemic and non-endemic areas. The p values with an asterisk show a significant difference between groups ($p<0.05$). NGP 2334: Galα1-3Galβ1-4GlcNAc-HAS, NGP 2333: Galα1-3Galβ1-3GlcNAc-HAS, NGP 2203: Galα1-3Gal-HAS, N.C.: β1-4-Galactosyl-Galactose-BSA (NGP 02040), P.C.: soluble proteins from crude extract from *Leishmania (Viannia) braziliensis* culture (strain MHOM/BR/84/LTB300), H1: healthy individuals from non-endemic areas, H2: healthy individuals from endemic areas, CL1: CL patients with active lesions and no treatment, CL2: CL patients with active lesions under the treatment, CL3: CL patients that had finished treatment and were under observation for the subsequent 3 months, OD: serum reagent patients for other diseases – such as Hepatitis B and C, Syphilis and truly positive patients for Tuberculosis, CD: serum reagent patients for Chagas disease.

			p values					
	Groups	**Positivity (%)**	**H2**	**CL1**	**CL2**	**CL3**	**OD**	**CD**
NGP 2334	**H1**	21.7	>0.9999	0.2477	0.0032*	<0.0001*	>0.9999	0.0496*
	H2	51.4		>0.9999	0.0645	0.0073*	0.3576	0.6020
	CL1	75.0			>0.9999	>0.9999	0.0625	>0.9999
	CL2	100.0				>0.9999	0.0008*	>0.9999
	CL3	100.0					<0.0001*	>0.9999
	OD	37.0						0.0190*
	CD	100.0						
NGP 2333	**H1**	34.8	>0.9999	0.1806	0.0201*	0.0005*	>0.9999	0.0071*
	H2	27.0		0.0375*	0.0059*	<0.0001*	>0.9999	0.0018*
	CL1	83.3			>0.9999	>0.9999	0.1294	>0.9999
	CL2	100.0				>0.9999	0.0167*	>0.9999
	CL3	100.0					0.0001*	>0.9999
	OD	35.2						0.0055*
	CD	100.0						

CONTINUE

			p values					
	Groups	**Positivity (%)**	**H2**	**CL1**	**CL2**	**CL3**	**OD**	**CD**
NGP 2203	**H1**	34.8	>0.9999	0.0205*	0.0089*	0.0004*	>0.9999	0.0037*
	H2	54.1		0.1769	0.0525	0.0047*	>0.9999	0.0237*
	CL1	91.7			>0.9999	>0.9999	0.1571	>0.9999
	CL2	100.0				>0.9999	0.0504	>0.9999
	CL3	92.3					0.0033*	>0.9999
	OD	46.3						0.0223*
	CD	100.0						
N.C.	**H1**	26.1	>0.9999	0.1310	0.2780	>0.9999	>0.9999	>0.9999
	H2	37.8		0.0547	0.1843	>0.9999	>0.9999	0.9806
	CL1	75.0			>0.9999	>0.9999	0.4284	>0.9999
	CL2	100.0				>0.9999	0.7235	>0.9999
	CL3	53.8					>0.9999	>0.9999
	OD	44.4						>0.9999
	CD	60.0						
P.C.	**H1**	17.4	>0.9999	0.0004*	0.0545	0.1902	>0.9999	0.0113*
	H2	16.2		<0.0001*	0.0099*	0.0160*	0.2131	0.0016*
	CL1	91.7			>0.9999	>0.9999	0.0063*	>0.9999
	CL2	80.0				>0.9999	0.3525	>0.9999
	CL3	61.5					>0.9999	>0.9999
	OD	40.7						0.0871
	CD	100.0						

Table 4. Mann-Whitney statistical analysis between groups of individuals from CL endemic and non-endemic areas and α-Gal antigens. The p values with an asterisk show a significant difference between groups ($p<0.05$). NGP 2334: Galα1-3Galβ1-4GlcNAc-HAS, NGP 2333: Galα1-3Galβ1-3GlcNAc-HAS, NGP 2203: Galα1-3Gal-HAS, NGP 0204: β1-4-Galactosyl-Galactose-BSA, *L. braziliensis*: soluble proteins from crude extract from *Leishmania (Viannia) braziliensis* culture (strain MHOM/BR/84/LTB300), H1: healthy individuals from non-endemic areas, H2: healthy individuals from endemic areas, CL1: CL patients with active lesions and no treatment, CL2: CL patients with active lesions under the treatment, CL3: CL patients that had finished treatment and were under observation for the subsequent 3 months, OD: serum reagent patients for other diseases – such as Hepatitis B and C, Syphilis and truly positive patients for Tuberculosis, CD: serum reagent patients for Chagas disease.

		p values	
	Antigen	***L. braziliensis***	**NGP 0204**
H1	**NGP2334**	0.0082*	0.0002*
	NGP 2333	0.4167	0.0299*
	NGP 2203	0.2921	0.0127*
H2	**NGP2334**	<0.0001*	<0.0001*
	NGP 2333	0.3025	0.1682
	NGP 2203	0.0010*	0.0031*
CL1	**NGP2334**	0.7430	0.0045*
	NGP 2333	0.1584	0.0679
	NGP 2203	0.4704	0.0100*
CL2	**NGP2334**	0.0079*	0.0159*
	NGP 2333	0.4127	0.2222
	NGP 2203	0.4127	0.0952
CL3	**NGP2334**	<0.0001*	<0.0001*
	NGP 2333	0.0101*	<0.0001*
	NGP 2203	0.0337*	<0.0001*
OD	**NGP2334**	0.2954	0.0121*
	NGP 2333	0.6829	0.0514
	NGP 2203	0.1497	0.0002*
CD	**NGP2334**	0.2222	0.0079*
	NGP 2333	0.3095	0.0079*
	NGP 2203	0.3095	0.0159*

Figures

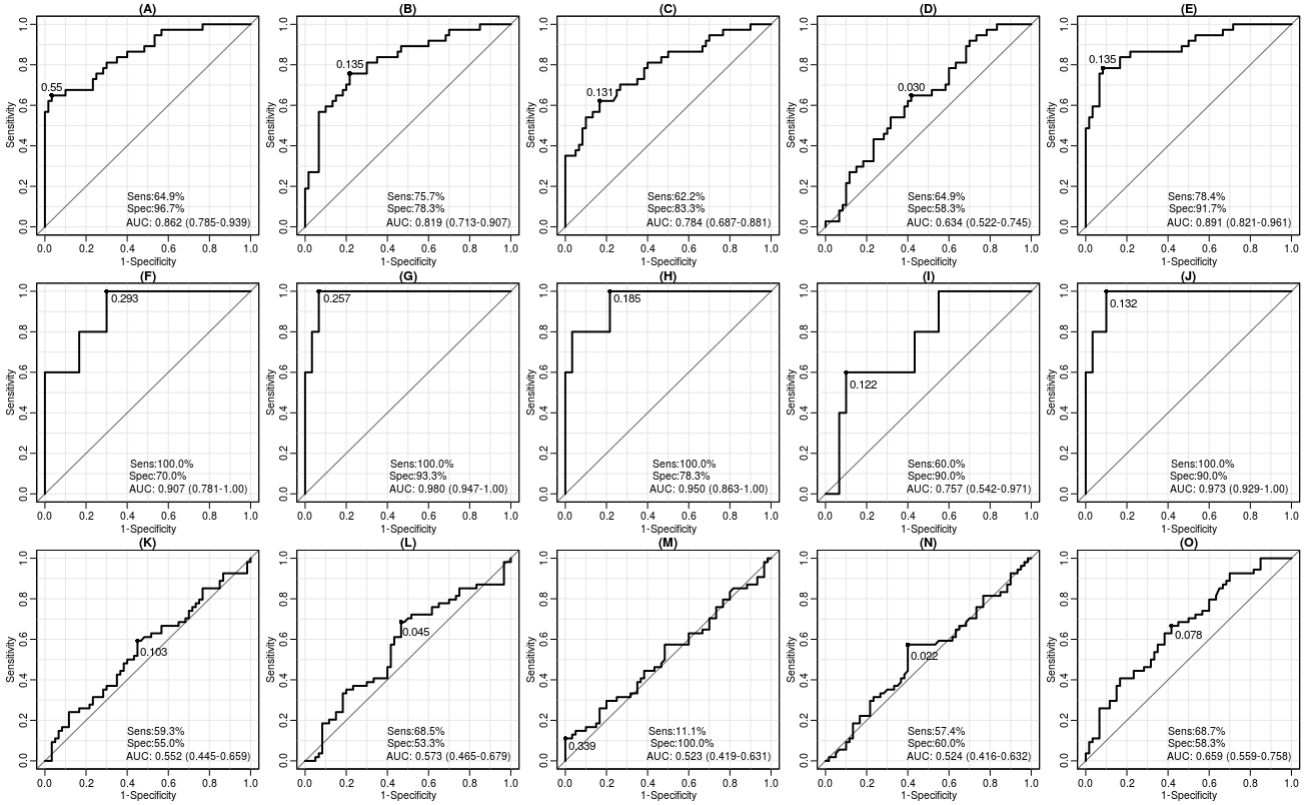

Figure 1. ROC curve analysis and sensitivity and specificity for NGP 2334, NGP 2333, NGP 2203 and NGP 0204 antigens compared to the ROC curve analysis for the positive control (*L. braziliensis*) for CL, Chagas disease and other diseases, performed using R software with auxiliary pROC system. NGP 2334: Galα1-3Galβ1-4GlcNAc-HAS, NGP 2333: Galα1-3Galβ1-3GlcNAc-HAS, NGP 2203: Galα1-3Gal-HAS, NGP 0204: β1-4-Galactosyl-Galactose-BSA, *L. braziliensis*: soluble proteins from crude extract from *Leishmania (Viannia) braziliensis* culture (strain MHOM/BR/84/LTB300), (A) NGP 2334 for CL, (B) NGP2333 for CL, (C) NGP2203 for CL, (D) - NGP0204 for CL, (E) *L. braziliensis* crude extract, (F) NGP2334 for Chagas disease, (G) NGP2333 for Chagas disease , (H) NGP2203 for Chagas disease, (I) NGP0204 for Chagas disease, (J) *L. braziliensis* crude extract for Chagas disease, (K) NGP2334 for other disease, (L) NGP2333 for other disease, (M) NGP2203 for other disease, (N) NGP0204 for other disease and *(*O) *L. braziliensis* crude extract for other disease.

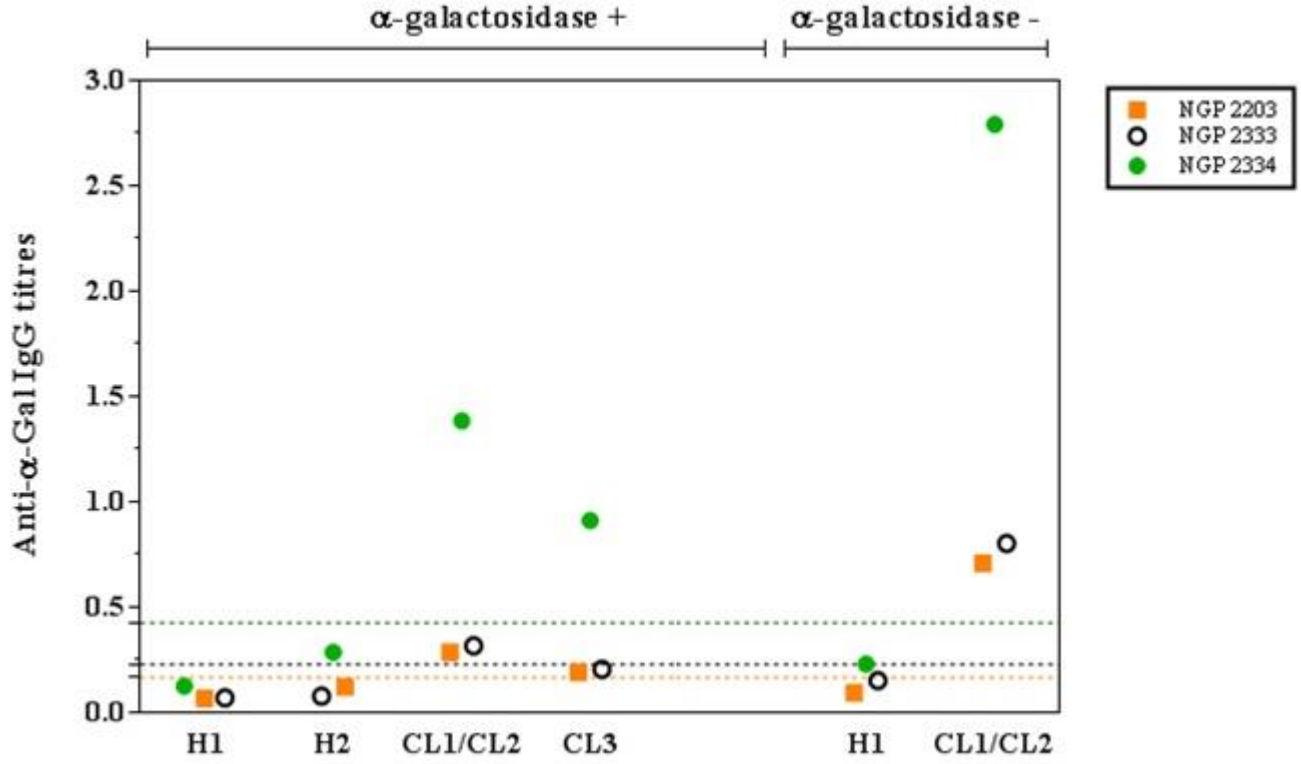

Figure 2. Levels of anti-α-Gal antibodies detected in individuals from CL endemic and non-endemic areas in Brazil after cleavage specific with α-galactosidase enzyme from Green Coffee beans on α-galactosylated NGPs antigen. NGP 2334: Galα1-3Galβ1-4GlcNAc-HAS, NGP 2333: Galα1-3Galβ1-3GlcNAc-HAS, NGP 2203: Galα1-3Gal-HAS, H1: healthy individuals from non-endemic areas, H2: healthy individuals from endemic areas, CL1: CL patients with active lesions and no treatment, CL2: CL patients with active lesions under the treatment, CL3: CL patients that had finished treatment and were under observation for the subsequent 3 months.