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Epidemiological, clinical and immune factors that influence the persistence of antiphospholipid antibodies in leprosy

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Abstract

Introduction: Antiphospholipid antibodies (aPL) are described in individuals with leprosy without the clinical features of antiphospholipid antibody syndrome (APS), a condition involving thromboembolic phenomena. We have described the persistence of these antibodies for over 5 years in patients with leprosy after specific treatment.

Objectives: To determine whether epidemiological, clinical and immunological factors played a role in the long-term persistence of aPL antibodies in leprosy patients after multidrug therapy (MDT) had finished.

Methods: The study sample consisted of 38 patients with a diagnosis of leprosy being followed up at the Dermatology and Venereology Outpatient Department at the Alfredo da Matta Foundation (FUAM) in Manaus, AM. ELISA was used to detect anticardiolipin (aCL) and anti-β_2 glycoprotein I (anti-β_2GPI) antibodies. Patients were reassessed on average of 5 years after specific treatment for the disease (MDT) had been completed.

Results: Persistence of aPL antibodies among the 38 leprosy patients was 84% (32/38), and all had the IgM isotype. Mean age was 48.1 ± 15.9 years, and 23 (72.0%) were male. The lepromatous form (LL) of leprosy was the most common ($n = 16$, 50%). Reactional episodes were observed in three patients (9.4%). Eighteen (47.37%) were still taking medication (prednisone and/or thalidomide). Mean IgM levels were 64 U/mL for aCL and 62 U/mL for anti-β_2GPI. In the multivariate binary logistic regression the following variables showed a significant association: age ($p = 0.045$, OR = 0.91 and CI 95% 0.82–0.98), LL clinical presention ($p = 0.034$; OR = 0.02 and CI 95% = 0.0–0.76) and bacterial index ($p = 0.044$; OR = 2.74 and CI 95% = 1.03–7.33). We did not find association between prednisone or thalidomide doses and positivity for aPL ($p = 0.504$ and $p = 0.670$, respectively). No differences in the variables vascular thrombosis, pregnancy morbidity, diabetes, smoking and alcoholism were found between aPL-positive and aPL-negative patients.

Conclusion: Persistence of positivity for aPL antibodies was influenced by age, clinical presentation and bacterial index. However, further studies are needed to elucidate the reason for this persistence, the role played by aPL antibodies in the disease and the B cell lineages responsible for generation of these antibodies.

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Introduction

Antiphospholipid (aPL) antibodies are autoreactive immunoglobulins that are very closely related to each other and react with anionic phospholipids. Thrombosis is the most important clinical event for aPL associated syndrome. Various studies have shown aPL antibody positivity in infectious diseases [1–7] or after exposure to some drugs [8]. Under these conditions, aPL are not usually associated with the clinical complications attributed to antiphospholipid antibody syndrome (APS) [9], are often short-lasting and can disappear when the infection is treated [1, 10]. Studies of leprosy patients with aPL failed to identify an association between the presence of these antibodies and thrombotic manifestations [1, 2, 5, 7, 11, 12].

Various studies have reported aCL antibodies in leprosy patients in frequencies varying from 20 to 98% using ELISA, primarily in lepromatous leprosy [5, 11, 13–20]. In tuberculoid forms the positivity is lower and varies between 7 and 39.5% [16, 20].

The literature on positivity for anti-β_2GPI antibodies in leprosy patients is conflicting. Some authors [15, 19], reported low positivity (2.9% until 18%), while others [1, 2, 5, 11, 12], report values from 39 to 89%. IgM was the isotype most frequently found by various authors [1, 2, 5]. However, in patients with the multibacillary LL form, other authors failed to find a predominant isotype [11].

While short-term positivity for the aPL has been observed in patients with infectious diseases such as infectious mononucleosis [6] and hepatitis B [4], persistent positivity has been reported in leprosy in two studies: in one, five out of six patients were positive for anti-β_2GPI antibodies for 2 years after their initial assessment [11], and in another, 32 patients who had completed MDT were positive for aPL antibodies for more than 5 years after their initial assessment [7]. The present study therefore sought to identify which epidemiological, clinical and immune factors contribute to persistence of these antibodies.

Materials & methods

Study population

The study sample consisted of 38 patients, previously described [7] with a diagnosis of leprosy and positive for one of the antiphospholipid antibodies and were followed up at the Dermatology and Venereology Outpatient Department at the Alfredo da Matta Foundation (FUAM) in Manaus, a referral center for treatment of leprosy in the state of Amazonas, and agreed to take part in the study. Serum samples and clinical data of these patients were collected in two occasions (June 2004 to October 2006 [7] and May 2010 to November 2011), when the patients were assessed.

Blood samples and laboratory tests

To carry out the tests to detect aPLs and identify their classes (IgG or IgM), 20 mL of peripheral venous blood was collected in dry tubes from all the participants. After centrifugation, the sera were aliquoted and frozen at − 20 °C in the FUAM laboratory. They were then sent to the rheumatology laboratory at the Federal University of São Paulo (UNIFESP) and the immunology laboratory at the Federal University of Amazonas (UFAM), where the tests were carried out. The sera collected and tested in 2004 were retested in 2012 together with the sera collected in 2010 and 2011 using the same kits for aCL and for anti-β_2GPI antibodies used previously to validate the results of the earlier tests.

Anticardiolipin antibodies (aCL)

ELISA was used to measure aCL with plates prepared in-house according to a standardized protocol used in the UNIFESP rheumatology laboratory. The ELISA plates (Polysorp NUNC, USA) were first sensitized with 50 μL/well of bovine heart cardiolipin (Sigma-Aldrich, St. Louis, MO, USA) dissolved in ethanol at a concentration of 50 μg/mL and kept overnight at 4 °C. They were then blocked for 1 h with 10% adult bovine serum albumin (BSA) in PBS (BSA/PBS). Next, test and control sera diluted 1:50 in BSA/PBS were added to duplicate wells (50 mL/well), and the plates were incubated overnight at 4 °C. After three washes in PBS, alkaline phosphatase-labeled anti-human IgG or IgM (Calbiochem, La Jolla, CA, USA) diluted 1:4000 and 1:5000, respectively, was added (50 mL/well). After incubation for 90 min at room temperature, the plates were washed, p-nitrophenyl phosphate (PNPP) was added (50 μL/well) and the plates were then kept at room temperature in the dark for 30 min. Absorbances were read at 450 nm using a plate reader/spectrophotometer (Labsystems Multiskan MS). A standard curve was constructed using international standards (LAPL-GM100 IgG/IgM Calibrators, Louisville APL Diagnostics Inc., Doraville, GA, USA) and the corresponding equation determined. The mean absorbances of the samples were inserted in this equation to get the results in IgG antiphospholipid (GPL) and IgM antiphospolipid (MPL) units. Values above 20 GPL and 10 MPL, respectively, were considered to indicate positivity for IgG and IgM anti-aCL antibodies. These reference values were obtained by calculating the 95th percentile of 200 blood donor samples analyzed at the UNIFESP rheumatology laboratory.

Anti-β_2-glycoprotein antibodies (anti-β2-GPI)

IgG and IgM anti-β_2-glycoprotein I (anti-β_2GPI) antibodies were detected by ELISA using commercial kits (BINDAZYME Human Anti-β_2GPI IgG and Anti-β_2GPI IgM, The Binding Site, Birmingham, UK) in accordance

with the manufacturer's instructions. The reaction was quenched by adding 100 mL of stop solution to each well, and absorbances were then read at 450 nm using a plate reader (Labsystems Multiskan MS).

A standard curve was plotted using the standards supplied with the kit, and the corresponding equation was then used to convert the absorbances of the samples to U/mL. Values above 20 U/mL and 10 U/mL, respectively, were considered to indicate positivity for IgG and IgM anti-β_2GPI antibodies.

Statistical analysis

The statistical analysis was performed with R version 2.14.0 (New Zealand). For the descriptive analysis the mean, median, standard deviation (SD) and minimum and maximum values were used for continuous variables and the proportions for categorical variables. The binomial test was used to assess differences in the distribution of the data.

To assess the possible factors that caused sera to test negative for aPL antibodies during the observation period, Pearson's chi-squared test was used for categorical variables and the Mann-Whitney test for continuous variables. Multivariate binary logistic regression was used in the first model, in which the dependent variable was the categorical variable aPL (1 = negative for aPL; 0 = positive for aPL) and the independent variables were gender (1 = male; 2 = female), age (continuous), lepromatous clinical presentation (1 = LL; 0 = other forms), prednisone use (1 = used; 0 = not used) and thalidomide use (1 = used, 0 = not used). In the second model, an adjustment was made for the different thalidomide or prednisone doses as continuous variables to determine whether they were associated with a change in reactivity from positive to negative. To assess whether the initial bacterial index affected positivity for the antibodies, binary logistic regression was used, with each antibody (aCL or anti-β_2GPI) included in the model as dependent variables and the initial bacterial index as the continuous independent variable. The Hosmer-Lemeshow test was used to assess the goodness of fit of the models. Logistic regression was performed with MiniTab® 16.

As the patients in this study were assessed at different times since they had been diagnosed with the disease, the hypothesis tested was that persistence of positivity for aPL would depend on the clinical presetnation of the disease (LL and the other forms, BT, BB and BV). The graphs were generated with GraphPad Prism 6.02.

Results

Patient clinical and demographic features

The study sample consisted of 38 patients with a diagnosis of leprosy and positive aPL. Mean age was 46.42 ± 16.62 years. Twenty-eight (73.6%) of the patients were male, and ten (26.3%) female. A test of proportions indicated that the difference in gender distribution was significant ($p = 0.005$). There are two main types of reactional episodes in Hansen patients. The Type 1 reactional episodes includes new skin lesions or neuritis and the Type 2 is mainly characterized by erythema nodosum leprosum (ENL). During the last clinical evaluation, no patients were observed with reactional episodes, however reactions were observed in four patients (10.5%) during the study. Among them four presented with erythema nodosum leprosum (ENL) and were taking thalidomide and prednisone, and one presented with neuritis and was taking prednisone (20 mg). Eighteen (47.37%) were using some medication, six were taking prednisone, four were taking thalidomide and eight both medications.

A significant difference was observed in the distribution of the clinical presentations in the 38 patients. The LL form was the most common clinical presentation ($p = 0.026$) and was found in 16 (50%) of the patients. The BT and BV forms were found in eight patients (25%) and seven patients (21.87%), respectively.

All 38 patients had completed MDT. Mean and median time between completion of MDT and blood collection was 87.00 and 67.50 months, respectively, with a maximum and minimum of 9 and 174 months. One patient had a relapse in 2009 but had already completed the treatment 9 months previously when the serum was collected. Mean and median time between the first and second collections were 66.89 and 66.10 months, respectively, with a minimum and maximum of 53.43 and 86.83 months.

Laboratory assessment of patients

The 38 sera positive for aPL antibodies in the first analysis (sera collected in 2004/2006) were retested at the same time with the sera collected in 2010/2011. All sera were tested for anti-β_2GPI antibodies, and for aCL antibodies (patients that were positive in 2004/2006). Of the retested sera, 37 (97.4%) remained positive for one of the aPLs and one (2.6%), who had previously been positive for anti-β_2GPI, was negative. At the new samples collected (2010/2011) 32 (84.2%) remained positive and six (15.8%) became negative.

aCL antibody was only detected in patients with the BV and LL clinical presentation. Of the nine patients positive for aCL antibody at the retest serum from 2004/2006, all of whom had the LL clinical presentation, two patients became negative in 2010/2011 sera. The anti-β_2GPI antibody was quantified in patients with the BT, BB, BV and LL clinical presentations both at the retest (serum from 2004) and in serum from 2010/2011. Positivity for this antibody was lower in the serum collected in 2010/2011 for all clinical presentations.

IgM was found more frequently than IgG in the three analyses of the sera. Of the nine patients retested for aCL antibody, seven were positive for IgM, and two for IgG and IgM. At the sera collected in 2010/2011, two became negative and seven remained positive for IgM. Of the 37 patients retested for the anti-β_2GPI antibody, 35 were positive for IgM, one was positive for IgG and IgM and one patient who had been positive for IgG became negative. Of the three patients who were positive for IgG and IgM, two became negative for IgG but remained positive for IgM. Thirty-one of the sera collected in 2010/2011 remained positive for anti-β_2GPI antibody, but only IgM was detected. Among patients positive for aCL antibodies the mean concentration of aCL antibodies was 74.3 U/mL in the retested serum in 2012 and 64.6 U/mL in the serum collected in 2010/2011. The concentrations of anti-β_2GPI antibodies were 67.7 and 62.5 U/mL, respectively.

Among nine patients positive for aCL antibodies at the retest, seven (77.8%) had IgM antibody titers > 40 U/mL (mean concentration = 88 MPL). Among seven, six (85.7%) still had IgM titers > 40 U/mL in 2010/2011 sera, with a mean aCL antibody concentration of 70.8 MPL. Anti-β_2GPI titers > 40 U/mL were found in 12/37 sera (32.4%) that were positive at the retest. One was IgG (159 U/mL), and 11 IgM. In the sera collected in 2010/2011 six of the 12 still had high concentrations.

Among patients positive for aPL antibodies with titers > 40 U/mL, the LL form was the predominant form among patients positive for anti-β_2GPI and aCL antibodies at the retest and in sera collected in 2010/2011. Among seven patients positive for aCL antibodies, six (85.7%) were also positive for anti-β_2GPI antibodies.

Considering the sera collected in 2010/2011, 32/38 (84.2%) were positive for aPL antibodies (aCL and anti-β_2GPI) and six (15.8%) were negative. Among 32, one was positive for the aCL antibody, 25 (78.1%) for the anti-β_2GPI antibody and six (18.8%) for both antibodies. We did not find difference on demographic and clinical characteristics between positive and negative individuals (Table 1). Similarly, no difference was observed regarding other clinical variables for these 38 patients between positive and negative individuals (Table 2). There was no association between demographic and clinical characteristics and titers for one of the aPL antibodies > 40 U/mL (eight – 25.0%) or ≤ 40 U/mL (24–75.0%), in the 32 patients. There was no difference in the frequencies of the clinical variables in patients with aPL antibody titers above 40 U/mL and in those with titers below 40 U/mL.

Table 3 shows that for the first multivariate binary logistic regression model (model 1), the variables age (p = 0.045, OR = 0.91 and 95% CI 0.82–0.98) and LL clinical presentation (p = 0.034; OR = 0.02 and 95% CI = 0.0–0.76) reduced the likelihood of patients becoming negative for aPL antibodies. The likelihood of older patients becoming negative for aPL antibodies is lower. Prednisone or thalidomide use and dose did not influence the change from positive to negative reactivity for aPL antibodies even when the model was adjusted for the doses of these two drugs (model 2) (p = 0.504; p = 0.670, respectively).

Table 1 Demographic and clinical characteristics of the 38 leprosy patients according to aPL positivity or negativity on sera collected in 2010/2011

Variables	Serum aPL (2010/2011 collected sera)						p
	Negative		Positive		Total		
	n	%	n	%	N	%	
Gender							0.670*
Male	5	83.3%	23	71.9%	28	73.7%	
Female	1	16.7%	9	28.1%	10	26.3%	
Age	37.0 ± 18.2		48.1 ± 16.0		46.3 ± 16.6		0.075**
Length of treatment	81.2 ± 18.3		77.4 ± 36.5		80.0 ± 34.1		0.389**
Clinical presentations							0.081*
BT	1	16.7%	8	25.0%	9	23.7%	
BB	2	33.3%	1	3.1%	3	7.9%	
BV	2	33.3%	7	21.9%	9	23.7%	
LL	1	16.7%	16	50.0%	17	44.7%	
Reactional episodes							1*
Present	0	0.0%	1	3.1%	3	7.9%	
Absent	6	100.0%	31	96.9%	35	92.1%	
Current reactional episodes							1*
Present	1	16.7%	3	9.4%	4	10.5%	
Absent	5	83.3%	29	90.6%	34	89.5%	
Medication							0.188*
No	5	83.3%	15	46.9%	20	52.6%	
Yes	1	16.7%	17	53.1%	18	47.4%	
Prednisone							0.278*
No	0	0.0%	4	23.5%	4	22.2%	
Yes	1	100.0%	13	76.5%	14	77.8%	
Thalidomide							0.387*
No	0	0.0%	6	35.3%	6	33.3%	
Yes	1	100.0%	11	64.7%	12	66.7%	

* Chi-square test ** Mann-Whitney Test

Table 4 shows that the initial bacterial index influenced positivity for aCL antibodies (p = 0.043; OR = 2.75) and anti-β_2GPI antibodies (p = 0.044; OR = 2.46). The initial (diagnostic) and current bacterial indices were grouped according to whether the patient was positive or negative for aPL antibodies using the Kruskal-Wallis test (p < 0.0001) and Dunn's test for multiple comparisons. Figure 1 shows that the diagnostic and initial bacterial index is greater in individuals positive for aPL antibodies (p = 0.029 and 0.012, respectively).

Table 2 Clinical variables for the 38 leprosy patients broken down according to aPL antibody positivity or negativity at the sera collected in 2010/2011

Variables	Serum aPL (2010/2011 collected sera)						*p*-value
	Negative		Positive		Total		
	N	%	n	%	N	%	
Vascular Thrombosis							1*
No	6	100.0%	31	96.9%	37	97.4%	
Yes	0	0.0%	1	3.1%	1	2.6%	
Pregnancy Morbidity							1*
No	6	100.0%	31	96.9%	37	97.4%	
Yes	0	0.0%	1	3.1%	1	2.6%	
Diabetes							1*
No	6	100.0%	30	93.8%	36	94.7%	
Yes	0	0.0%	2	6.3%	2	5.3%	
Smoking							0.670*
No	3	50.0%	20	62.5%	23	60.5%	
Yes	3	50.0%	12	37.5%	15	29.5%	
Alcoholism							0.315*
No	5	83.3%	31	96.9%	36	94.7%	
Yes	1	16.7%	1	3.1%	2	5.3%	

*Chi-square test

Discussion

The sample in this study consisted of outpatients with various forms of leprosy at a referral center for leprosy in the state of Amazonas. Most of the subjects had lepromatous leprosy, did not present with reactions and had completed specific MDT a long time previously (mean 78.0 months). The interval between the first and second blood collection was 66.9 months on average.

Table 3 Results of multivariate binary logistic regression to assess probable factors causing leprosy patients to become negative for aPL antibodies

Predictor	95% CI	Odds ratio (OR)	*p*-value
Model 1			
Age	0.82–0.98	0.91	0.045
Gender	0.0–2.84	0.11	0.185
Lepromatous form	0.0–0.76	0.02	0.034
Prednisone use	0.0–4.88	0.09	0.237
Thalidomide use	0.01–14.7	0.44	0.647
Model 2			
Age	0.84–0.97	0.92	0.044
Female	0.0–2.16	0.08	0.134
Lepromatous form	0.0–0.92	0.03	0.044
Prednisone dose	0.71–1.18	0.92	0.504
Thalidomide dose	0.95–1.03	0.99	0.670

Table 4 Results of binary logistic regression to assess the influence of initial bacterial index on presence of aPL antibodies in 38 leprosy patients

Model	95% CI	Odds ratio (OR)	*p*-value
aCL vs. Initial Bacterial Index 1	1.03–7.33	2.75	0.043
Anti-β_2GPI vs. Initial Bacterial Index 2	1.02–5.58	2.46	0.044
aPL vs. Initial Bacterial Index 3	1.03–7.33	2.74	0.044

According to some authors, aPL antibodies in leprosy are often transient and can disappear after treatment [1, 10]. However, in the previous study of our group, in which 158 leprosy patients were followed up, positivity for aPL antibodies was not affected by time since completion of MDT, and titers remained high for months and years afterwards. No evidence was found to suggest that the presence of aPL antibodies is a transient phenomenon [5]. A longitudinal study with these patients was therefore undertaken to assess the persistence of these autoantibodies, which was confirmed by the findings of the study [7]. In the present study, several factors that could contribute to this finding, such as the epidemiological, clinical and immune characteristics of the disease, were investigated comparing patients that were positive or negative for aPL antibodies. The predominance of males among the patients in our study agrees with the literature, according to which detection rates in most countries apart from some countries in Africa are twice as high in men as in women [21].

Because there was a trend toward a statistically significant association between some variables, such as clinical presentation ($p = 0.081$), age ($p = 0.075$) and treatment ($p = 0.188$), when $p < 0.2$ was used to indicate a trend toward statistical significance [22], we performed multiple logistic regression adjusted for gender. This revealed that persistence of positivity for aPL antibodies was influenced independently by age, bacterial index and the LL clinical presentation. The data show that more advanced age tends to increase the likelihood of the patient being positive for aPL antibodies, possibly because of cumulative exposure to antigens of mycobacteria and other bacteria or because of immune system aging, which prevents complete elimination of *M. leprae*. Persistence was observed predominantly in leprosy patients with the LL form, whose sera collected in 2010/2011 had titers greater than 40 U/mL (77.8% for aCL and 32.4% for anti-β_2GPI).

The persistence of the IgM isotype suggests the T-cell independent nature of the response to these non-peptide antigens, which are recognized by B1 B and MZ B (splenic marginal-zone B) cells. These antigens generally

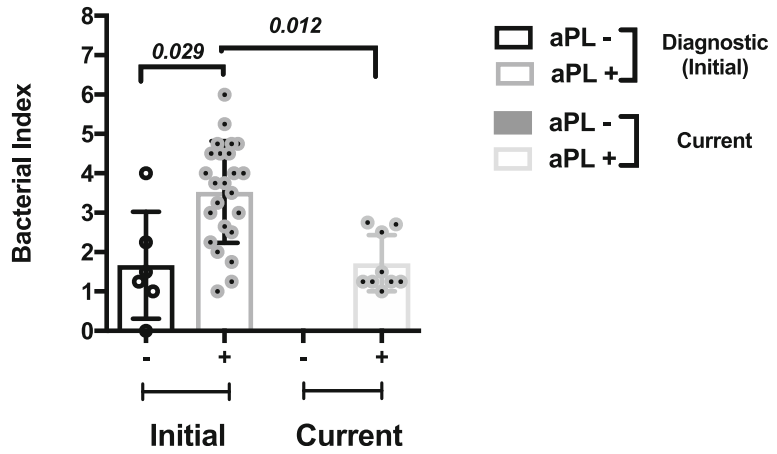

Fig. 1 Initial bacterial index (2004) and current bacterial index (2010/2011) according to positivity or negativity for aPL antibodies in 38 leprosy patients

include sugars and glycolipids that induce production of low-affinity IgM antibodies and rarely induce a class switch to IgG. Cardiolipin is a membrane phospholipid found in bacterial (including mycobacterial) membrane. Thus, it is probable that in the LL clinical presentations, which has a higher bacterial index, the *M. leprae* membrane phospholipids are recognized by B1 or MZ B cells as extracellular phase antigens at the bloodstream. Interestingly, the complement system can be activated by the presence of M. leprae in tissues, leading to the production of C3d, which is required for B-cell activation. It is also curious that bacteria in the intestine and urinary tract such as *Escherichia coli* contain cardiolipin in their membrane, suggesting that natural IgM aCL antibodies can be found in healthy individuals [23, 24].

Our results strongly indicate that the initial bacterial index influenced positivity for aPL antibodies. It is therefore reasonable to suppose that *M. leprae* persists in latent, or inactive, form in the human body even after treatment, particularly in individuals with the LL clinical presentations, who supposedly have a Th2 response. Latency is an acknowledged phenomenon in infections by mycobacteria, as suggested by the reactivation of tuberculosis when a "cured" infected person is severely immunosuppressed [25]. Spirochetes, bacteria of the genus Borrelia and viruses, particularly those in the family Herpesviridae, can also exhibit latency [26, 27]. The presence of reactional episodes and prolonged use of prednisone or thalidomide support persistence of the pathogen, albeit in inactive form, and suggest discrete interaction with the immune system. A noteworthy finding of the present study is that the treatment did not influence positivity and that the most important factor appears to be the bacterial load.

Some potential limitation of this study should be noted. Since this was an observational study in design, the inference and causality are limited. Moreover, some marginal associations may not be significant due to the relatively small number of patients retested. New information was recorded for the patients that lost follow-up and were not evaluated for a second time. This could affect the sensitivity of the study to detect thrombosis and APS. However, the incidence rate for APS is expected to low for adults over 18-years old as 2.1% [28], and this probably did not affect the present results.

Conclusion

As in APS, IgM aPL antibodies are common in leprosy patients and persist for long periods in these individuals but without thromboembolic manifestations. Further studies are needed to elucidate the reason for this persistence, the role played by aPL antibodies in the disease and the B-lymphocyte lineages responsible for production of these antibodies. This knowledge would help to improve our understanding of the immunological mechanisms involved in the interaction between *M. leprae* and the human host.

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Nothing to declare.

Authors' contributions

SLER design of the project, main responsible by ethics approval and data collection. HLAP: was co-responsible by data collection and design of the project. ALB: data analysis and interpretation. NPS: laboratory assays. EIS, NPS, LFSP and MCS: design of the project. SLER, ALB, MCS: writing of the manuscript. All authors read and approved the final manuscript.

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Availability of data and materials

All data and materials of this manuscript are available for from the corresponding author on reasonable request.

Ethics approval and consent to participate
Patients signed an Informed Consent Form, and the study was approved by the Ethics Committee of the Fundação Alfredo da Matta No. 008–10.

Consent for publication
Not applicable.

Competing interests
The authors declare that they have no competing interests.

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