Anti-oxidized LDL antibodies as atherosclerosis development markers in HIV patients undergoing antiretroviral therapy: a longitudinal cohort study

K. R. O. M. Ronchini, H. Goto, A. J. S. Duarte, and M. Gidlund

Abstract—Antiretroviral therapy (ART) drastically improved the life expectancy and quality of Human Immunodeficiency Virus (HIV)-infected patients, but ART causes adverse effects that include metabolic alterations considered risk factors for atherosclerosis that need monitoring. Anti-oxidized LDL (oxLDL) antibodies with known role in atherosclerosis may be used as a marker to monitor its development. A longitudinal cohort study for 12 months involved Naïve patients (n=20) with no ART, and ART patients (n=51) that initiated ART during the study period. CD4 T cell counts, viral load, lipid profile, anti-oxLDL and anti-apolipoprotein B-derived peptide D antibodies and carotid intima-media thickness (IMT) were evaluated. The ART group signifcantly recovered the CD4 T cell count (P<0.001) and lowered the viral load (P<0.001) while the Naïve group maintained their levels throughout. Anti-oxLDL and anti-apoB-D antibody levels were correlated (r=0.657, P<0.001), and were similar between groups at time zero. They progressively decreased in the ART group in the 12-month period but there was no change in the Naïve group. At 12 months, a carotid IMT was evaluated in 18 patients undergoing ART, and it was found above or close to the upper normal value in all. We found an inverse correlation between carotid IMT and anti-apoB-D (r=-0.410, P=0.045) and anti-oxLDL (r=-0.392, P=0.054) antibody levels. Antiretroviral therapy improved the parameters of HIV infection, but it may have induced increased carotid IMT. Its inverse correlation mainly with anti-apoB-D antibody level strengthens this antibody level trend as a marker of atherosclerosis development in HIV patients under antiretroviral therapy.

Submitted:
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Index Terms—apoB peptide, ART, atherosclerosis, HIV, lipodystrophy, oxLDL

I. INTRODUCTION

ANTIRETROVIRAL therapy (ART) has drastically changed the rapid, progressive clinical outcome in Human Immunodeficiency Virus (HIV)-infected patients, increasing their life expectancy. However, adverse events related to the use of ART have arisen, and an alarming and increasing number of patients develop metabolic alterations that constitute risk factors to atherosclerotic disease [1] [2] that may ensue ischemic coronary artery disease or cerebral ischemia [3] [4].

The atherosclerotic process leading to plaque rupture and thrombus formation is initiated by an inflammatory event and the formation of lipid-laden macrophages in the vascular subendothelial space [5]. Methods to evaluate the risk of developing atherosclerosis or to assess the current status in a patient are available. Amongst the non-invasive methods, the Framingham score is the most commonly used [6] [7]. A more direct method is the measurement of carotid intima-media thickness (IMT), and it is widely used in observational studies [8] [9] [10] [11] [12] [13]. It would however be of interest to develop an alternative, easy method to evaluate atherosclerosis development.

Several mechanisms are involved in the development of atherosclerosis. The presence of immune cells in the plaque and the strong and continuous immune response against modified Low Density Lipoprotein (mLDL) that includes oxidized LDL (oxLDL) imply that the immune system is involved in the atherosclerotic process [14]. In particular, circulating antibodies against mLDL have been associated with either protection from or aggravation of the disease [15] [16] [17] [18] [19] [20] [21] [22] [23]. Therefore, we have here done a prospective study of HIV patients receiving ART to evaluate its implication in atherosclerosis development and to assess the possibility of using antibodies against mLDL or selected peptides for assessment and follow-up in patients at risk for cardiovascular complications.

In the present study, the patients were followed for 12 months, and the clinical status and classical risk factors for developing
atherosclerosis were analyzed. The antibodies against oxLDL were evaluated, both the polyclonal response against oxLDL and the clonal response against one synthetic peptide D derived from apolipoprotein B (apoB). The antibody response against this apoB-derived peptide D (apoB-D) was well correlated to the polyclonal response against mLDL [24], avoiding the intra-experimental differences between batches of mLDL. We found a decay in the antibody levels against mLDL and apoB-D, suggesting a subsequent and gradual increase in the circulating and tissue-fixed mLDL. Most interestingly, using the apoB-D peptide, a negative correlation to carotid IMT was found in randomly selected patients.

II. METHODS

A. Objectives

The main focus of this work was to evaluate if antibodies against oxLDL could serve as a direct- or pseudo-marker for increased risk for atherosclerosis development in HIV-positive patients undergoing ART. This hypothesis was tested evaluating anti-oxLDL and anti-apolipoprotein B-derived peptide D antibodies in a longitudinal cohort study for 12 months involving patients with no ART (Naïve), and ART patients that initiated ART during the study. At the end point, antibody levels were correlated with carotid IMT.

B. Study Design and Participants

This is a longitudinal cohort study for 12 months. Patients were selected from among HIV-1-infected individuals that have been followed at the HIV outpatient service of the Hospital das Clínicas da Faculdade de Medicina da Universidade de São Paulo and Instituto de Infectologia Emílio Ribas, São Paulo, Brazil. Ethical committees from both institutions approved the protocol and written, informed consent was obtained from all participants. Patients were more than 18 years old and were included if they had no recent or current infections in the three-month period before enrollment and no use of corticosteroids. Seventy-one HIV-positive patients were enrolled from April 2002 through June 2004 and followed up for at least 12 months. The demographical data, self-reported HIV infection transmission route, tobacco and alcohol use, and HIV disease status according to the 1993 Centers for Disease Control and Prevention (CDC) classification [25] were obtained using a questionnaire at the initial visit. During clinical evaluation, hypertension was defined as a systolic pressure ≥ 140 mmHg or a diastolic pressure ≥ 90 mmHg or current use of blood pressure lowering drugs for hypertension. Diabetes was considered if glucose levels were above ≥ 7 mmol/L after two measurements or the use of medication for diabetes.

The patients were divided in two groups. One group consisted of patients that had not reached the criteria for ART during the follow-up period (Naïve group; n=20). The other group consisted of patients that started antiretroviral treatment within the period of the study (ART group; n=51), and they were included in this group at the moment of the introduction of ART. Patients of both groups were followed for at least 12 months, assessed at least every 4 months for HIV infection, immunological status evaluation and blood sampling. Drugs were prescribed according to the Brazilian guidelines in use at that time [26], and the patients in the present study used different regimens: 11 (21.6%) patients used two Nucleoside Reverse Transcriptase Inhibitors plus one Non-Nucleoside Reverse Transcriptase Inhibitor, 27 (52.9%) used two Nucleoside Reverse Transcriptase Inhibitors plus one Protease Inhibitor and 13 (25.5%) used two Nucleoside Reverse Transcriptase Inhibitors plus one boosted Protease Inhibitor as the first-line regimen. Patients had been in therapy for at least 12 months. Blood samples were collected at time zero (before the introduction of ART) considered baseline data and every four months during the 12 months for lipid fractions and glucose evaluation. Serum samples were stored at -70°C until the analysis of anti-oxidized LDL (oxLDL) and anti-apoB-D antibodies.

C. CD4+ T Lymphocyte Cell Count and Viral Load Evaluation

CD4+ T Lymphocyte cell count in peripheral blood was determined by Becton-Dickinson immunocytometry system (San Jose, Calif., USA) using BD TruCount System and HIV RNA viral load by Nucleic Acid Sequence Based Amplification (NASBA) using the kit NUCLESENS HIV-1 QT–BioMérieux which detection range is from 80 to 10,000,000 copies/mL.

D. Lipid Profile and Glucose

Total cholesterol, high density lipoprotein (HDL)-cholesterol and triglycerides were determined using enzymatic methods in a Cobas Integra® automatic analyzer (Roche Diagnostics). LDL-cholesterol was calculated by the Friedwald equation. Glucose was measured by the oxidase method.

E. Detection of Antibodies Against oxLDL and apoB-D

Fractionation of LDL, preparation of oxLDL for use as an antigen in the antibody detection assay, and an ELISA assay to detect anti-oxLDL and anti-apoB-D antibody levels were done as in Kethelhuth et al. [27]. To detect anti-apoB-D antibodies, the plates were coated using 1 µg/mL solution. The results were expressed as Reactivity Index (RI) to be able to compare different plates analyzed on different days because more than 500 samples were evaluated. An IgG control with protein concentration of 0.116 µg/mL in phosphate buffered saline 0.01M, pH 7.2 (PBS) and PBS were used as a control. Antibodies against oxLDL and apoB-D values are shown in relative numbers using the formula RI = (Sample absorbance – PBS absorbance)/ (control IgG absorbance).
F. Carotid IMT Measurements

Ultrasound color-Doppler exam was performed by one physician specifically trained to evaluate carotid vessels, specifically the epi-aortic vessels, using a Philips HDI 5000 power color-Doppler with 5 – 12 MHz probes (Bothel, WA, USA). Characteristics of the common carotid artery, internal carotid artery, and carotid sinus IMT were evaluated and the greater thickness measurement was recorded for both sides. An IMT > 1.00 mm was considered pathological. Atherosclerotic plaques, if present, were also recorded.

G. Statistical Analysis

Statistical analyses were performed using GraphPad Prism3 (GraphPad Software, Inc., San Diego, CA, USA) using either an ANOVA test with the Student Newman–Keuls contrast post-test or Mann-Whitney. Regression and Pearson correlation were also applied. Differences were considered significant when P<0.05.

III. RESULTS

The present longitudinal cohort study involved 71 HIV-positive patients divided into a Naïve group (n=20) and an ART group (n=51), predominately composed of males with no race distribution differences. The patients in the Naïve group were significantly younger than those in the ART group (P<0.05) (Table 1). In most cases, the HIV was sexually transmitted. In Table 1, the demographic distribution as to HIV disease classification is also given, as well as time from diagnosis and known risk factors for coronary artery disease, i.e., smoking, hypertension, and diabetes.

Initially, we analyzed the groups under treatment separately by considering the types of drug in use, either Non-Nucleoside Reverse Transcriptase Inhibitor or Protease Inhibitor. However, this analysis showed no differences regarding the parameters evaluated; we therefore analyzed all the patients in a single group. We started analyzing parameters related to the HIV infection by evaluating the viral load and the CD4+ T cell count as an indication of immunological status (Table 2). When the patients were enrolled in the study (time zero), median CD4+ T lymphocyte counts were significantly lower in the ART than in the Naïve group, 226 and 787 cells/µL, respectively (P<0.001). In the Naïve group, this parameter did not change significantly during the 12-month follow-up, but in the ART group it increased 85.4, 171.6 and 208.4% in 4, 8 and 12 months, respectively, reaching 398 cells/µL in the latest period (Table 2, P<0.001). Mean HIV viral load was significantly higher in the ART group than in the Naïve group.

### Table 1

<table>
<thead>
<tr>
<th>Variables</th>
<th>Groups</th>
<th>Naïve</th>
<th>ART</th>
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</thead>
<tbody>
<tr>
<td>Age (years)</td>
<td></td>
<td>33 ± 10</td>
<td>39 ± 7*</td>
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<tr>
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<td>24:57</td>
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<td>8(40.0)%</td>
<td>14(27.5)%</td>
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<td>A</td>
<td></td>
<td>17(85.0)%</td>
<td>12(23.5)%</td>
</tr>
<tr>
<td>B</td>
<td></td>
<td>3(15.0)%</td>
<td>22(43.1)%</td>
</tr>
<tr>
<td>C</td>
<td></td>
<td>17(51.0)%</td>
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<td>Time from HIV diagnosis (months)</td>
<td></td>
<td>14(0)(12)*</td>
<td>24(0)(30)</td>
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</table>

n = number of patients, ART = antiretroviral therapy
* mean ± standard deviation, ** n(%) , † according to Centers for Disease Control classification for HIV infection
*P<0.05 (Mann-Whitney test)

### Table 2

| CD4+ CELL COUNT, HIV VIRAL LOAD, LIPID PROFILE AND GLUCOSE AT BASELINE AND 12TH MONTH |
|---------------------------------|-----------------|
| Groups                          |                 |
| Naïve (n=20)                    | ART (n=51)      |
|                                | Baseline 12th Month Baseline 12th Month |
| CD4+ cell count (cells/µL), median | 787 (449:2141)* | 639 (428:730) | 226 (6:359)** | 398 (78:650)* |
| Viral Load (log10), log mean ± SD | 3.95 ± 0.72 | 3.87 ± 1.08 | 4.73 ± 0.76** | 0.63 ± 1.48* |
| Triglycerides (mmol/L), median | 1.2 (0.6:2.8) | 1.2 (0.6:2.6) | 1.5 (0.7:6.8) | 1.8 (0.7:6.8)** |
| Total cholesterol (mmol/L), median | 4.1 (1.8:5.8) | 4.2 (1.7:5.4) | 4.2 (2.5:6.3) | 5.2 (3.1:11.9)** |
| LDL cholesterol (mmol/L), median | 2.6 (1.4:4.6) | 2.6 (1.3:3.6) | 2.6 (0.8:4.2) | 2.8 (1.8:4.5) |
| HDL cholesterol (mmol/L), median | 1.0 (0.2:1.6) | 1.0 (0.6:1.3) | 0.9 (0.3:3.5) | 1.2 (0.7:1.8) |
| Glucose (mmol/L), median | 4.5 (3.6:5.4) | 4.6 (3.4:5.6) | 4.8 (3.8:6.0) | 5.0 (3.6:6.7) |

$= (minimum:maximum), SD = standard deviation, ART = Antiretroviral therapy
L DL = low-density lipoprotein, HDL = high-density lipoprotein
*P<0.001 in relation to baseline ART
**P<0.001 in relation to baseline Naïve
*P<0.05 in relation to 12 months Naïve (ANOVA and Newman–Keuls Tests)
at time zero (P<0.001). In the Naïve group, this parameter did not change significantly after 12 months, but in the ART group, it decreased 55.6, 83.8 and 85.6% after 4, 8 and 12 months, respectively (Table 2, P<0.001).

Serum triglycerides, total cholesterol, LDL and HDL levels were determined during follow-up (Table 2). No difference was found between the Naïve and ART groups at time zero (P>0.05). In the ART group, we found fluctuations in lipoprotein fraction profiles and glucose levels individually but no significant changes in the levels after 12 months (P>0.05), except for triglycerides and total cholesterol, where a significant increase was seen after 12 months (P<0.001). In the Naïve group, no significant alterations were seen in these parameters.

Concerning antibody levels, the anti-oxLDL antibody levels were not significantly different between groups at time zero (data not shown) but the anti-apoB-D antibody levels were lower in ART compared with Naïve group, respectively, 0.26 (0.11:0.43) [median (minimum:maximum)] and 0.34 (0.21:0.69) RI. During follow-up we observed a progressive decrease in the anti-oxLDL and anti-apoB-D antibody levels in the ART group (Figs. 1A and 1B). No alterations were seen in anti-oxLDL level during the follow-up period in the Naïve group (data not shown). Anti-oxLDL and anti-apoB-D antibody levels showed a significant positive correlation (r=0.657, P<0.001) (Fig. 1C).

Although CD4+ T cell count had increased with treatment and anti-oxLDL and anti-apoB-D antibody levels decreased in the ART group as a whole, we observed individual variations and we analyzed whether antibody level variation was related to CD4+ T cell count. When we analyzed the antibody levels separately, according to the CD4+ T cell count, we observed that the decrease in the antibody levels with treatment were independent of the CD4+ T cell count of the patients (Fig. 2).

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**Fig. 1.** Levels of anti-oxLDL (A) and anti-apoB-D (B) antibodies by ELISA in HIV patients under antiretroviral therapy (ART) at 0, 4, 8, and 12 months (n = 51) and correlation between anti-oxLDL and anti-apoB-D antibody levels (C). ELISA data presented as Reactivity Index (RI), horizontal bars = median, n = number of patients, *** P<0.001 (ANOVA and Newman-Keuls Multiple Comparison Test), # P<0.0001 (Regression Analysis)

**Fig. 2.** Levels of anti-oxLDL (A and B) and anti-apoB-D (C and D) antibody by ELISA in Naïve (A and C) and antiretroviral therapy (ART) (B and D) groups according to different CD4+ T lymphocyte counts by flow cytometry. Antibody levels presented as Reactivity Index (RI), horizontal bars = median, n = number of patients, Naïve group - n = 20, ART group - n = 51, *** P<0.001 in relation to baseline ART (Mann-Whitney test)
In some randomly selected patients (n=18), we measured the carotid artery IMT at the 12th month of follow-up using Doppler. Five were treated with Nucleoside Reverse Transcriptase Inhibitors and 13 with Protease Inhibitor. Considering the segment with the greater thickness, 2 of the 18 patients (11.1%) presented thickness greater than 1.00 mm (threshold normal value), and the mean value (0.89 mm) in all other patients was close to the upper limit. No difference was seen when the data were analyzed considering therapeutic regimen, smoking, hypertension and diabetes. However, when we related this parameter of all 18 patients to the anti-oxLDL or anti-apoB-D levels, a negative correlation between carotid IMT and anti-oxLDL antibody level was found (P=0.054). Further and most interestingly, a significant negative correlation was found between carotid IMT and anti-apoB-D antibody level (P=0.045) (Fig. 3).

IV. DISCUSSION

The main focus of this work was to evaluate if antibodies against oxLDL, a well-known component in atherogenesis, could serve as a direct- or pseudo-marker for increased risk for atherosclerosis development in HIV-positive patients undergoing ART.

Antibodies and their roles have been extensively explored, and the current view is that they can either protect or aggravate the disease [15] [16] [17] [18] [19] [20] [21] [22] [23]. A large part of the disagreement about the role of anti-oxidized LDL antibodies is caused by the huge number of antigenic and chemically different epitopes present on oxLDL or modified LDL.

To in part overcome these problems in this study, we have evaluated the response against both the non-defined oxLDL particle and one selected peptide epitope from the apoB, apoB-D [24]. Using this peptide we diminished the large heterogeneity in the antibody pool.

The composition of the ART and Naïve groups was similar for most of the demographic data. There was a significant difference in age; however, analysis excluding those patients that differed in age range showed that this factor did not interfere with the results. Another parameter that was distinct between the groups as expected was in CDC classification, where most of the patients of the Naïve group were classified in group A. This was expected, as this is related to the initiation of the ART therapy. The groups did not differ in exposure to risk factors to atherosclerosis, i.e., tobacco use, hypertension and diabetes.

In the evaluation of the parameters related to HIV infection evolution, CD4+ T cell count in the patients of the Naïve group was higher than the ART group, as expected. This was maintained throughout the 12-month duration of this study, and therefore these patients did not receive any antiretroviral therapy. In the patients of the ART group, the CD4+ T cell count was low in the beginning and there was an increase after 12 months of treatment, but still in a lower level than the Naïve group.

Levels of glucose and lipoproteins were evaluated as their alterations are considered major risk factors for atherosclerosis. We did not observe significant changes in the fasting glucose level of patients under treatment, which may be because the length of the study was not long enough. However and most interestingly, we found early alterations in the lipid profile. At the end of the 12-month treatment, 17 (34.7%) patients developed hypertriglyceridemia and 10 (20.4%) developed hypercholesterolemia in the ART group.

The analysis of anti-apoB-D antibodies showed lower levels in the patients that started antiretroviral therapy compared to those without treatment. In ART group both anti-oxLDL and anti-apoB-D antibody levels reduced gradually during 12 month follow-up period, despite at least partial immune reconstitution evaluated by CD4+ T lymphocyte count. Antibody levels that were higher in Naïve patients did not show any significant change during the 12-month follow-up.

We found a good but not complete correlation between anti-apoB-D and anti-oxLDL antibodies that somehow was expected. However, it clearly shows that the apoB-D peptide contains at least one immunodominant epitope that is promiscuous. This peptide is localized in the inner part of the apoB that cover the LDL particle [28]. Thus, this epitope can
only be exposed in LDL particles subjected to oxidation or other types of decomposition.

An explanation for the gradual decay of anti-oxLDL and anti-Apo B peptide D antibody levels may be their consumption/neutralization by the generation of ox-LDL or mLDL generated during antiretroviral therapy. Support for this comes from the report that there is an inverse correlation between antibody levels and circulating or increased tissue-fixed mLDL [29]. Recent studies by us have shown that altered levels of reactivity against oxLDL or apoB-D are associated with the presence of acute coronary disease or exposure to environmental factors or genetic predisposition for development of atherosclerosis [21] [24] [30]. Another explanation could be a diminished capacity to produce anti-mLDL antibodies or a lack of certain anti-mLDL specificities [16] [27].

The finding of a highly significant drop in antibody reactivity against oxLDL and a more pronounced drop using the apoB-D peptide led us to analyze carotid IMT using Doppler. It has been shown that HIV-infected patients or those under treatment have increased carotid IMT [10] [11] [31] [32] [33]. In the present study however, we considered the time scale of 12 months to be very short for doing a follow-up of the carotid IMT. Thus, we evaluated this parameter at the end point in some patients and we correlated it with the anti-mLDL response. The analysis of the carotid IMT revealed a tendency for an inverse correlation with the anti-oxLDL antibody level but a significant inverse correlation with anti-apoB-D peptide antibody levels. The explanation for this could be that the ongoing inflammation/immune response against the increased HIV circulation or tissue-fixed mLDL causes a reduction of available free antibodies. This increase is influenced by the medication in comparison to the naïve patients. In such a scenario, it is predictable that the fluctuation in antibody levels is far more rapid than the process leading to detectable increase in the carotid IMT. Thus, the reduced antibody levels that are more easily detected would precede any detectable increase of the carotid IMT and may be used to presume the pathological condition associated to atherosclerosis. However, we are aware that further studies in experimental systems and patient follow-up studies will have to confirm the present findings.

V. CONCLUSION

In conclusion, we have found that antiretroviral therapy in patients showing a decrease in viral load and partial reconstitution of the immune system demonstrates a decrease in the level of antibodies against oxLDL and an apoB-D peptide. We suggest that this decay can be associated with the increase in atherosclerosis development seen in these patients. This could occur in HIV-infected patients, and as shown here, it is more pronounced in patients undergoing ART. The inverse correlation found between the immune-dominant response against one single neo-epitope of apoB only expressed during modification and carotid IMT using eco-Doppler may in the future result in methods to follow or screen carotid artery disease patients using this far less expensive methodology.

LIST OF ABBREVIATIONS

Anti-apoB-D: anti-apolipoprotein B-derived peptide D; ART: antiretroviral therapy; CDC: Centers for Disease Control and Prevention; ELISA: Enzyme-Linked Immunosorbent Assay; HDL: High Density Lipoprotein; HIV: Human Immunodeficiency Virus; IgG: Immunoglobulin G; IMT: intima-media thickness; LDL: Low Density Lipoprotein; mLDL: modified Low Density Lipoprotein; oxLDL: oxidized LDL; PBS: phosphate-buffered saline; RI: reactivity index.

CONFLICTS OF INTEREST

The authors declare that they have no financial or non-financial competing interests.

AUTHOR’S CONTRIBUTIONS

KROMR and MG conceived the project and defined the focus of the study and MG coordinated all development of the study. KROMR assisted the patients, obtained and recorded their clinical and laboratory parameters, performed the anti-oxLDL and anti-apoB-D peptide antibody determinations, and done statistical analysis. AJSD provided clinical information of patients that he supervised which samples were included in the initial studies. KROMR, HG and MG analyzed the results step by step and wrote the manuscript. All authors have read, revised and approved the manuscript.

ACKNOWLEDGMENT

This study was supported by Fundação de Amparo à Pesquisa do Estado de São Paulo (research grant to MG and fellowship to KROMR), Instituto Nacional de Ciencia e Tecnologia – INAMI, and Conselho Nacional de Pesquisa (research fellowships to MG and HG). We acknowledge the personnel of the Ambulatório de Imunodeficiência Secundária “ADEE – 3002” HC-FMUSP (Casseb J, Gonzalez C, Almeida A, Mendonça M, Veiga AP, Bueno A), and of Instituto de Infectologia Emílio Ribas (Lindoso AL and Novoa P) for support on medical assistance of the patients, the researchers of the Laboratório de Imunofisiopatologia, ICB-USP (Ketelhut DF, Rios FO) for technical advice, the researcher of Instituto de Medicina Tropical, USP, Sanchez, MCA for full text and table editing and Silvana Silva for technical assistance.

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