Serum antiphospholipid antibody levels as biomarkers for diagnosis of pulmonary tuberculosis patients

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SUMMARY

SETTING: Salvador, Bahia, Brazil.

OBJECTIVE: To evaluate the immunoglobulin (Ig)M and total IgG antibody response to cardiolipin (CL), phosphatidylcholine (PTC), phosphatidylethanolamine (PE), phosphatidylinositol (PI) and sulfatide (SL-I) as biosignatures that can be used to diagnose pulmonary tuberculosis (TB) and its applicability for monitoring the efficacy of anti-tuberculosis treatment.

DESIGN: Serum samples from 37 adult pulmonary TB patients and 48 controls (16 healthy household contacts, 19 household contacts with latent tuberculous infection [LTBI] and 13 non-TB patients with lung disease) were screened using enzyme-linked immunosorbent assays (ELISAs) for IgM and total IgG against phospholipids.

RESULTS: Levels of IgM response to CL, PE and PI, and IgG response to CL, PE, PI and PTC were significantly higher in TB patients than in control groups. Anti-CL IgG had the best performance characteristics, with a sensitivity and specificity of respectively 86.5% and 87.2%. This IgG anti-CL ELISA test detected 86.5% (32/37) of the TB patients, whereas the number detected using sputum smear was only 65.9% (24/37). After anti-tuberculosis treatment, the median value for all anti-phospholipid antibodies decreased significantly compared with baseline values (P < 0.05).

CONCLUSION: Our results suggest that the total IgG anti-CL level could be useful to complement conventional bacteriological tests for the rapid diagnosis of adult pulmonary TB.

KEY WORDS: cardiolipin; phosphatidylcholine; phosphatidylethanolamine; phosphatidylinositol; sulfatide

MYCOBACTERIUM TUBERCULOSIS kills approximately 2 million people each year and is estimated to latently infect one third of the world’s population.1 The early diagnosis and adequate treatment of pulmonary tuberculosis (PTB) patients are considered essential to reduce transmission of M. tuberculosis and to achieve the World Health Organization’s (WHO’s) End TB goals.2 The development of a real-time polymerase chain reaction assay for the detection of M. tuberculosis DNA and the mutations associated with resistance to rifampicin has been the most important advance in TB diagnostics in the last few years.3 However, the need for sophisticated laboratory infrastructure and highly skilled laboratory technicians remains a major barrier to the test’s introduction in low- and middle-income countries.4 Furthermore, the test does not eliminate the need for conventional tests, which are required to monitor response to treatment and relapse status.

Alternatives to traditional methods designed to directly detect M. tuberculosis are serological tests. Serological tests are simple, economical and minimally invasive, and can be used to diagnose TB smear-negative patients. However, the WHO has not endorsed serological testing based on targeting M. tuberculosis proteins due to their low sensitivity and specificity.5,6

The mycobacterial cell wall has unique features consisting of various lipids comprising >40% of the cell wall dry weight. These lipids can behave as antigens capable of stimulating specific B-cells to produce immunoglobulins (Igs).7 They have gained vital importance in recent years mainly due to modern approaches of lipidomic analysis. IgG- or IgM-mediated responses against mycobacterial phospholipids may therefore constitute a clinically useful tool for presumptive diagnosis and discrimination of PTB from other pulmonary diseases.8,9 Lipids abundant in M. tuberculosis include lipoarabinomannan (LAM), mycolic acids, phenolic glycolipids, polyacyltrehalose and lipooligosaccharides.10 Other components of the cell wall are cardiolipin (CL), phosphatidylglycerol, phosphatidylcholine (PTC), phosphatidylinositol (PI) and basic phospholipids such as phosphatidyletha-
nolamine (PE) and sulfatide (SL-I). These phospholipids have a dynamic structure involved in regulation of the transport of nutrients, toxic host-cell effector molecules and anti-tuberculosis drugs.11 The aim of the present study was to evaluate IgM and total IgG antibody responses against CL, PE, PI, PTC and SL-I to assess their potential as biomarkers for the diagnosis of PTB and in monitoring treatment of patients with PTB.

METHODS

Subjects and setting
A total of 85 subjects from 6o Centro de Saúde Rodrigo Argolo, Salvador, BA, Brazil, were enrolled in a cross-sectional study conducted between January 2012 and October 2013. Participants were categorised as PTB patients or controls. The control groups comprised healthy subjects with no evidence of infection, healthy subjects with LTBI and non-TB patients with other pulmonary diseases. Patients and controls who tested positive for human immunodeficiency virus and patients taking immunosuppressive drugs were excluded. All patients and all controls provided written informed consent. The study protocol was approved by the Human Subject Ethics Committee of Instituto Gonçalo Moniz, Salvador, BA, Brazil (IGM; Fundação Oswaldo Cruz).

Pulmonary tuberculosis patients
Thirty-seven patients with clinical symptoms suggestive of TB and one or more of the following characteristics were selected: 1) chest radiography (CXR) suggestive of TB opacities, 2) sputum samples that contained acid-fast bacilli (AFB) on microscopy; and 3) response to anti-tuberculosis drugs. Sputum smear microscopy was performed using Ziehl-Neelsen staining; results were grouped as negative, 1+, 2+ or 3+.12

Household contacts
Thirty-five individuals who had been exposed to TB patients during the patients’ symptomatic period were invited to participate in the study. Active PTB was excluded from household contacts (HHCs) using CXR and sputum smear. This group included only HHCs who underwent both the tuberculin skin test (TST) and interferon-gamma release assays (IGRAs). The TST was carried out using the Mantoux procedure with 2 tuberculin units of RT23 purified protein derivative (PPD) (Statens Serum Institute, Copenhagen, Denmark). Reading was performed after 72 h and categorised as: 0–5 mm, negative; >5 mm, positive and indicative of infection by M. tuberculosis. For IGRAs, we used QuantiFERON®-TB Gold In-Tube (QFT-GIT; Qiagen, Hilden, Germany). The test was performed according to the manufacturer’s instructions.13 The cut-off value for a positive response was 0.35 international units (IU)/ml. The HHCs were stratified into two groups: 16 were TST- and IGRA-negative (HHC−) and 19 were TST- and IGRA-positive (HHC with LTBI, HHC+).

Non-tuberculosis patients
Thirteen patients with lung diseases other than TB, who declared that they did not have any contact with patients with PTB, were used as a control group. TB was excluded using sputum smear, CXR and culture. Among these patients, 5 had bacterial pneumonia, 2 had lung cancer, 1 had bronchial asthma and the remaining 5 subjects had other pulmonary infections.

Enzyme-linked immunosorbent assay
Serum specimens were obtained upon recruitment and stored at −80°C until tested. Patients who had a confirmed diagnosis of TB received the standard treatment in Brazil and serum samples were prospectively collected at baseline as well as at 2 and 6 months after starting treatment.

Total IgG and IgM levels were measured using an indirect enzyme-linked immunosorbent assay (ELISA).14 Lipids were diluted to 10 mg/ml (CL, PE, PI and PTC) or 1 mg/ml (SL-I) (Sigma-Aldrich, Saint Louis, MI, USA) using anhydrous ethanol; 50 µl of the solution was then added to each well of polystyrene ELISA plates (Greiner Bio-One, Kremsmünster, Austria). The plates were covered with a plate sealer (Costar™; Corning, Wiesbaden, Germany) and incubated overnight (18–24 h) at room temperature. The coated wells were washed once with 1× phosphate-buffered saline (PBS), pH 7.4 (1× PBS; Invitrogen, San Diego, CA, USA), blocked with 100 µl of 3% low fatty acid bovine serum albumin (BSA) (blocking buffer), and incubated for 1 h at room temperature. Plates were washed twice using 300 µl 1× PBS, and 100 µl of the serum sample diluted 1:100 in 3% BSA was added. After incubation for 1 h at room temperature, the serum samples were removed and the plates were washed thrice with 1× PBS. Then, respectively 100 µl of 1:10 000 and 1:50 000 goat-derived anti-human IgM and total IgG, labelled with horseradish peroxidase (Sigma-Aldrich) diluted in 3% BSA/PBS, was added. After 1 h of incubation, a new cycle of washes and 100 µl/well of the chromogenic substrate tetramethylbenzidine (Invitrogen) was added, and the reaction stopped with 100 µl of 2N sulphuric acid. Reactions were read within 10 min at 450 nm in a spectrophotometer (Thermo Fisher Scientific, Waltham, MA, USA). A group of three wells in each ELISA plate did not receive any samples; these blank wells were used as negative controls. Results were read out as the average optical density (OD) of triplicate samples and were re-run if >10% coefficient of variance was observed.
TB patients were separated by degree of smear status,

0.06; Figure 1 A4, A5). It should be noted that when
ostatistical significance used a level of
non-TB patients as the control group. All tests for
together with the controls. We defined HHCs and
and specificity based on samples from the patients,
resulting in the combination of the highest sensitivity
constructed for the two anti-phospholipid antibody
Receiver operating characteristic (ROC) curves were
anti-tuberculosis treatment, differences between
ney U-test. For longitudinal analysis of Ig levels on
comparisons were analysed using the Mann–Whit-

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\text{Isotype Phospholipid} \quad \text{AUC (95%CI)} \quad \text{P value} \quad \text{Sensitivity} \quad \text{Specificity} \quad \text{LR}^+ \\
\text{IgM} \quad \text{CL} \quad 0.742 (0.636–0.848) \quad 0.0001 \quad 70.3 (53–84.1) \quad 68.8 (53.8–81.3) \quad 2.2 \\
\quad \text{PE} \quad 0.762 (0.658–0.865) \quad <0.0001 \quad 75.7 (58.8–88.2) \quad 70.8 (55.9–83) \quad 2.6 \\
\quad \text{PI} \quad 0.836 (0.748–0.923) \quad <0.0001 \quad 77.8 (60.8–89.9) \quad 79.2 (65–89.5) \quad 3.7 \\
\text{IgG} \quad \text{CL} \quad 0.940 (0.890–0.989) \quad <0.0001 \quad 86.5 (71.2–95.5) \quad 87.2 (74.26–95.2) \quad 6.8 \\
\quad \text{PE} \quad 0.782 (0.671–0.894) \quad <0.0001 \quad 72.2 (54.8–85.8) \quad 78.7 (64.3–89.3) \quad 3.4 \\
\quad \text{PI} \quad 0.880 (0.808–0.953) \quad <0.0001 \quad 81.1 (64.8–92) \quad 79.2 (65–89.5) \quad 3.9 \\
\quad \text{PTC} \quad 0.829 (0.737–0.921) \quad <0.0001 \quad 75 (57.8–87.9) \quad 75 (60.4–86.4) \quad 3.0 \\
\]

AUC = area under the receiver operating characteristic curve; CI = confidence interval; LR = likelihood ratio; + = positive; CL = cardiolipin; PE = phosphatidylethanolamine; PI = phosphatidylinositol; PTC = phosphatidylycholine.

**Statistical analysis**

Statistical analyses were performed using Prism v7.0 (GraphPad, San Diego, CA, USA). For analysis of antibody levels, differences between the three participant groups were first assessed using the Kruskal–Wallis test, followed by the Dunn’s post-test. If significance was found (\( P < 0.05 \)), pairwise comparisons were analysed using the Mann–Whitney U-test. For longitudinal analysis of Ig levels on anti-tuberculosis treatment, differences between time points were assessed using the Friedman test. Receiver operating characteristic (ROC) curves were constructed for the two anti-phospholipid antibody isotypes to identify cut-offs according to the value resulting in the combination of the highest sensitivity and specificity based on samples from the patients, together with the controls. We defined HHCs and non-TB patients as the control group. All tests for statistical significance used a level of \( P < 0.05 \).

**RESULTS**

**Characteristics of study subjects**

A total of 85 subjects volunteered to participate in our study. Of 37 (43.5%) PTB patients, 24 (64.9%) had sputum samples that were smear-positive for AFB. From these TB patients’ households, 16 healthy controls (HHC–) and 19 individuals with LTBI (HHC+) were also included. In addition to these groups, we included 13 subjects with lung disease other than TB. Characteristics of the study population have been described elsewhere.15

**IgM and total IgG antibody responses to CL, PE, PI, PTC and SL-I phospholipids in active TB patients at baseline**

Serum levels of total IgG and IgM specific to CL, PE, PI, PTC and SL-I phospholipids were evaluated in active TB patients at their first visit to the out-patient clinic before treatment. IgM response to CL, PE and PI was consistently higher in TB patients than in the control groups (\( P = 0.0014, P = 0.0002 \) and \( P < 0.0001 \), respectively, Figure 1 A1–A3). No difference was observed in PTC (\( P = 0.231 \)) or SL-I levels (\( P = 0.06; \) Figure 1 A4, A5). It should be noted that when TB patients were separated by degree of smear status, TB patients with negative sputum smear tests had statistically higher levels of anti-CL IgM (\( P = 0.023 \)) and anti-PI (\( P = 0.012 \)) than sputum smear-positive TB patients (data not shown).

Moreover, the IgG antibody response to CL, PE, PI and PTC was also increased (all phospholipids \( P < 0.0001 \)) in TB patients compared with controls (Figure 1 B1–B4). However, the median difference in levels of total serum IgG (\( P = 0.430 \)), as well as IgM to SL-I, was not statistically significant between TB patients compared with controls (Figure 1 A5). There were no statistically significant differences in the median IgG levels by sputum smear status (data not shown). Furthermore, there was no significant difference in changes in antibody levels between patients with cavitary PTB and those with non-cavitary PTB (\( P > 0.05; \) data not shown).

**IgM and total IgG test**

The ROC analysis indicated that PI performed better diagnostically in measuring IgM than CL and PE (Figure 2, Table). Conversely, CL had a better diagnostic performance in measuring IgG than PE, PI and PTC (Figure 3, Table). Total IgG had the best overall performance; on optimal ROC analysis, the IgG anti-CL ELISA test detected 86.5% (32 of 37) of TB patients (Figure 4), whereas the number detected using sputum smear was only 64.9% (24/37). Of the 32 IgG test-positive patients, 87.5% (21/25) were positive among AFB smear-positive patients. Anti-CL IgG ELISA thus identified 11 (84.6%) additional cases among 13 who were sputum smear-negative. When anti-CL and anti-PI IgG test results were considered, the number of positive results was not higher in TB patients; thus, the combined results did not improve the sensitivity of any test.

**Time-course changes in IgG and IgM antibody titres after initiation of anti-tuberculosis treatment**

To assess whether determination of phospholipid antibody responses could be useful for monitoring the efficacy of anti-tuberculosis treatment, a series of serum samples was collected from active TB patients who had undergone anti-tuberculosis treatment. The median levels of IgM to CL and PI, IgG to CL, PE, PI...
Figure 1  Serum IgM (A1–A5) and total IgG (B1–B5) levels against CL, PE, PI, PTC and SL-I phospholipids in TB patients (n = 37), HHCs (n = 35), HHC− (n = 16), HHC+ (n = 19) and non-TB patients (n = 13). Statistical significance was determined using the Kruskal–Wallis test, followed by the Dunn test if significance was found (P < 0.05); pairwise comparisons were analysed using the Mann–Whitney U-test. * P < 0.05; ** P < 0.01; *** P < 0.001 and **** P < 0.0001. Horizontal
and PTC decreased significantly during anti-tuberculosis treatment (Figure 5). In addition, there was no statistically significant difference between median levels of IgM to CL and HHCs or non-TB patients ($P = 0.059$). With regard to the other phospholipids, even after treatment completion and despite reduction in median antibody levels, TB patients continued to have higher levels of IgM to PE ($P = 0.0006$) and PI ($P = 0.0001$) or IgG to CL ($P < 0.0001$), PE ($P = 0.038$), PI ($P = 0.002$) and PTC ($P = 0.019$) than HHCs or non-TB patients.

**DISCUSSION**

We observed that antiphospholipid antibody levels, including anti-CL, anti-PE, anti-PI and anti-PTC, were significantly higher in new adult PTB patients than in adult control groups. The IgG test had the best performance. The prevalence of detection of TB patients using the total IgG test (CL, 86.5% and PI, 81.1%) was higher than that detected using sputum smear results (AFB 64.9%) or the IgM test (CL, 70.3% and PI, 77.8%). In post-primary PTB patients,
low titres of IgM and high titres of IgG could explain the high rates of IgG positivity. Antiphospholipid IgG antibodies could thus be useful in complementing conventional bacteriological tests for rapid diagnosis and in discriminating PTB from other pulmonary diseases in patients presenting with similar symptomatology. Although the Xpert® MTB/RIF assay (Cepheid, Sunnyvale, CA, USA) is not available at Centro de Saúde Rodrigo Argolo, results should be discussed in the light of other diagnostic tests that are much more sensitive than sputum smear microscopy.

Although the role of M. tuberculosis cell wall phospholipids during infection is uncertain, CL is essential for M. tuberculosis growth in vitro. Antibodies against M. tuberculosis CL have also been...
found in serum samples from TB patients in other studies.\textsuperscript{18,19} In line with our results, higher levels of anti-CL antibodies were observed in TB patients than in other control groups before treatment.\textsuperscript{19,20} However, anti-CL antibodies are also considered to be auto-antibodies produced by the immune system that non-specifically target the body’s CLs, which are found almost exclusively in the inner mitochondrial membrane.\textsuperscript{21}

As anti-CL antibody levels in TB patients have rarely been investigated, it is not clear if these antibodies represent auto-antibodies or result from polyclonal B cell activation after stimulation by mycobacterial CLs. In the present study, the phospholipids used in the assay were purchased from a commercial company and were therefore not extracted from \textit{M. tuberculosis} cell wall lipids. Although the results do not reflect a ‘real’ response, the increase in levels of antiphospholipid antibodies in TB patients cannot be disregarded. If the anti-CL antibodies include auto-antibodies, these test results should be interpreted with caution based on the corresponding patient clinical symptoms and signs. No patients reported any symptoms that could be attributable to autoimmune disease, particularly those volunteers who had increased levels of anti-CL antibodies. Although the absence of symptoms does not exclude autoimmune disease, it is probable that antibody antiphospholipids are involved in TB disease. We therefore hypothesised that phospholipids released from lung cells during massive necrotic cell death induced by some strains of \textit{M. tuberculosis} in a patient could be recognised by the immune system as antigens, contributing to the increased production of auto-antibodies. Furthermore, the presence of auto-antibodies to antigens that are not mycobacterial, such as anti-Ro, anti-ß2-glycoprotein and anti-mitochondrial antibody, as negative controls could also be very helpful.

Goodridge et al. observed that in TB patients with cavitary pulmonary disease, the anti-lipid IgM response did not decrease with treatment.\textsuperscript{14} In our study, 16/28 (57.1\%) TB patients had cavitary disease. Anti-CL IgG levels decreased significantly after anti-tuberculosis treatment \textit{(P = 0.003)} in patients with cavitary lung disease. In contrast, the IgM levels did not fall \textit{(P = 0.144)}, probably because large amounts of lipids from both dead lung cells and \textit{M. tuberculosis} are released, inducing IgM antiphospholipid antibody production.

Our findings suggest that increased anti-CL antibody levels found in TB patients were not observed in other pulmonary conditions that induce inflammation (other types of bacterial pneumonia, asthma, chronic obstructive lung disease). Thus, despite the non-specific nature of the anti-lipid IgM and IgG responses to CL, the test appears to demonstrate a high degree of specificity if applied to pulmonary diseases.

IgM and IgG levels against all anti-phospholipid antibodies significantly decreased following anti-tuberculosis treatment. Similarly, Goodridge et al. showed that levels of IgM antiphospholipid antibodies decreased among non-cavitary TB patients.\textsuperscript{14} However, despite the reduction in Ig levels, our ELISA did not perform as well as the bacteriological test in monitoring response to treatment. This may have been due to the inclusion of TB patients with cavitary disease in our study. The data confirm the observations of Horne et al., who demonstrated that bacteriological tests remain useful for monitoring anti-tuberculosis treatment.\textsuperscript{22}

Compared with assays based on cell-mediated responses, antibody detection is considerably simpler and less expensive. However, serological tests still have suboptimal sensitivity and specificity. In the present study, the sensitivity of the IgG test to CL was high (86.5\%) compared with the IgM test (77.8\% to PI). The sensitivity for most of the lipids reported in other studies was low in TB patients compared with our findings.\textsuperscript{23,24} Furthermore, the specificity of the IgG anti-CL ELISA test (87.2\%) showed satisfactory results compared with other tests based on lipid antigens.\textsuperscript{24}

\begin{figure}[h]
\centering
\includegraphics[width=\textwidth]{Figure5.png}
\caption{Plot of the median change and interquartile range in A) IgM antiphospholipid and B) IgG antiphospholipid antibody levels before, during and after anti-tuberculosis treatment. All 37 patients were followed up for 24 weeks, and blood samples were collected before treatment (baseline) as well as after 8 and 24 weeks of treatment. OD = optical density; Ig = immunoglobulin; CL = cardiolipin; PE = phosphatidylethanolamine; PI = phosphatidylinositol; PTC = phosphatidylcholine. This image can be viewed online in colour at http://www.ingentaconnect.com/content/iuatld/ijtld/2018/00000022/00000009/art00016}\end{figure}
Finally, the sample size in our study was small; studies with large sample size and longitudinal follow-up, particularly among HHCs infected by *M. tuberculosis*, are required to understand the role of the antiphospholipid antibody in the development of TB disease. It is also important to validate the study in other cohorts with more subjects with clinically indistinguishable disease, such as bacterial pneumonia, from those who have TB and perform IGRAs in non-TB patients. IGRAs are not widely available to test non-TB patients in Brazil.

**CONCLUSION**

The WHO does not recommend serological tests for the diagnosis of TB based on protein antigens, particularly due to their lack of accuracy. This is likely because the humoral response to *M. tuberculosis* in chronic infection is highly complex and variable. However, based on our results, the clinical utility of serological tests against *M. tuberculosis* phospholipids has potential utility as a rapid screening test for differentiating TB from non-TB pulmonary diseases.

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Conflicts of interest: none declared.

**References**

CONTEXTE : Salvador, Bahia, Brésil.
OBJECTIF : Evaluer la réponse en anticorps immunoglobuline (Ig) M et en IgG totales à la cardiolipine (CL), à la phosphatidylcholine (PTC), à la phosphatidyléthanolamine (PE), au phosphatidylinositol (PI) et au sulfatide (SL-I) comme une biosignature qui peut être utilisée pour le diagnostic de la tuberculose (TB) pulmonaire et son applicabilité au suivi de l’efficacité du traitement antituberculeux.

SCHEMA : Des échantillons de sérum de 37 patients adultes atteints de TB pulmonaire et de 48 témoins dont 16 contacts domiciliaires en bonne santé, 19 contacts domiciliaires atteints d’infection tuberculeuse latente et 13 patients non-TB mais atteints de maladie pulmonaire, ont été dépistés par titrage avec immunoadsorbant lié à une enzyme (ELISA) à la recherche d’IgM et d’IgG totales vis-à-vis des phospholipides.

RESULTATS : Les niveaux d’IgM en réponse à CL, PE et PI et d’IgG en réponse à CL, PE, PI et PTC ont été significativement plus élevés chez les patients TB que dans les groupes témoins. Les IgG anti-CL ont eu les meilleures caractéristiques de performance avec une sensibilité et une spécificité de 86,5% et 87,2%, respectivement. Ce test ELISA d’IgG anti-CL a détecté 86,5% (32/37) des patients TB, tandis que le nombre détecté par frottis de crachats a été de seulement 65,9% (24/37). Après traitement antituberculeux, la médiane pour tous les anticorps antiphospholipides a significativement diminué, comparée aux valeurs de départ (P < 0,05).

CONCLUSION : Nos résultats suggèrent que les IgG totales anti-CL pourraient être utilisés pour compléter les tests bactériologiques conventionnels pour le diagnostic rapide de la TB pulmonaire des adultes.