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Changes in serum amyloid A, plasma high-density lipoprotein cholesterol and apolipoprotein A-I as useful biomarkers for *Mycobacterium tuberculosis* infection

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Abstract

Introduction. In recent years, cholesterol has received interest in the study of infection due to evidence of a relationship between low plasma cholesterol levels and tuberculosis (TB).

Hypothesis/Gap Statement. Plasma lipid profiles of serum amyloid A (SAA), apolipoprotein A-I and high-density lipoprotein cholesterol (HDL-C) are biomarkers associated with symptomatic TB patients.

Objective. We aimed to evaluate plasma lipid profiles of apolipoprotein A-I, SAA and the size of HDL as biomarkers to diagnose symptomatic TB patients.

Methodology. Patients with TB symptoms attending the Instituto Brasileiro para a Investigação da Tuberculose/Fundação José Silveira (IBIT/FJS) between September 2015 and August 2016 for diagnosis of TB were studied. From 129 patients, 97 were classified as pulmonary TB and 32 as negative-bacilloscopy (non-TB group). Medical history, fasting serum and plasma were obtained. Total cholesterol (TC), HDL-C, apolipoprotein A-I and SAA were measured by enzymatic or immunochemical reaction assays. HDL size was measured by laser light-scattering.

Results. In TB patients, TC (147.0±37 vs. 168±44 mg dL^{-1}), HDL-C (37±14 vs. 55±18 mg dL^{-1}) and apolipoprotein A-I (102±41 vs. 156±47 mg dL^{-1}) concentrations were lower (P<0.0001), while HDL particle size (10.16±1.02 vs. 9.62±0.67 nm) and SAA levels (280±36 vs. 19±8 mg L^{-1}) were higher (P<0.0001). Using receiver-operating characteristic curve analysis for predicting TB, the cutoff values were <83.85 mg L^{-1} for SAA (sensitivity=96.88%, specificity=78.43%, P<0.0001), >44.50 mg dL^{-1} for HDL-C (sensitivity=75%, specificity=72.16%, P<0.001) and >118.5 mg dL^{-1} for apolipoprotein A-I (sensitivity=83.83%, specificity=72.22%, P<0.001).

Conclusion. SAA, HDL-C and apolipoprotein A-I are associated with TB infection and could be used as laboratory biomarkers, especially in patients who are negative for alcohol-acid-resistant bacilli.

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Keywords: apolipoprotein A-I; HDL-C; lipoprotein diameter; serum amyloid A; tuberculosis-associated biomarkers.

Abbreviations: AFB, acid-fast bacilli; apo, apolipoprotein; AUC, area under the curve; CI, confidence interval; CRP, C-reactive protein; HDL, high-density lipoprotein; HDL-C, high-density lipoprotein cholesterol; LDL, low-density lipoprotein; LDL-C, low-density lipoprotein cholesterol; LPS, lipopolysaccharide; LR, likelihood ratio; ROC, receiver operating characteristic; SAA, serum amyloid A; TB, tuberculosis; TC, total cholesterol; VLDL, very low-density lipoprotein; VLDL-C, very low-density lipoprotein cholesterol.

INTRODUCTION

Mycobacterium tuberculosis is the causative agent of tuberculosis (TB), an infectious disease mainly affecting the lungs. Most individuals contacting this mycobacterial disease will be asymptomatic, but around 10% will go on to develop the disease [1]. TB remains a serious public health problem, currently representing the third highest cause of death due to infectious diseases, and it is the leading cause of death among human immunodeficiency virus-infected/AIDS patients. Among the 22 countries with the highest number of cases and disease burden, Brazil is in position 18, with 67000 diagnosed cases and 4400 deaths annually [2].

The most common diagnostic method for *M. tuberculosis* is sputum assaying acid-fast bacilli (AFB), being fast and low cost, the drawback being low sensitivity and a lack of ability to differentiate *Mycobacterium* species. The second most commonly used method is solid medium culture, which is still considered low cost and has higher sensitivity and specificity [3] than the sputum smear but requires a much longer time to reach a diagnosis (3–4 weeks). Despite being a faster method, liquid culture requires high-cost diagnostic equipment and laboratory infrastructure, thus restricting its use [4]. In 2010, the World Health Organization approved the Xpert MTB/RIF for TB diagnosis, and it was subsequently introduced in Brazil in 2014. The Xpert MTB/RIF, a nucleic acid amplification technique, is used in cases of naïve suspected TB, but it is not used to monitor patients under treatment since the test can remain positive in the absence of bacterial viability. To monitor treatment response, sputum smear microscopy is still used [5].

Biomarkers from blood, sputum, urine and pleural effusions have been proposed as infection flags, but no diagnostic test is currently approved for definitive diagnosis [6]. Recently, Akpovi *et al.* [7] showed a relationship between low plasma cholesterol concentration and infectious diseases, including TB. Several circulating lipid markers, such as lipid peroxidation species, were shown to be increased in pulmonary TB. Moreover, adequate cholesterol concentrations, lipoprotein-cholesterol, are necessary for regulating the immune system against infections [8–11]. In addition, in a case report, Sasaki *et al.* [12] described that untreated TB infection decreases serum lipid levels.

Changes in serum lipoprotein cholesterol in pulmonary TB patients are closely associated with important metabolic alterations [13]. The lipoproteins have constitutive components with both structural and metabolic characteristics, such as apolipoprotein (apo) A-I, apolipoprotein B, paraoxonase, phospholipid transfer protein, cholesterol ester transfer protein, lecithin:cholesterol acyltransferase, lipoprotein lipase and hepatic lipase, conferring particle stability and remodeling, and having antioxidant action.

Levels *et al.* [14] showed a similar association between phospholipid transfer protein and lipopolysaccharide (LPS)-binding protein. They also explored phospholipid transfer protein LPS transport to high- and low-density lipoprotein (HDL and LDL). This transport activity provides modified lipoprotein formation, typically observed in some types of dyslipidemia, such as that observed in acute phase responses in infectious diseases. It appears that HDL behaves as an immediate protection factor by adsorbing/transporting LPS until the cellular immune system is properly activated [15]. On the other hand, although HDL is the highest affinity lipoprotein for LPS, LDL and very low-density lipoprotein (VLDL) are also known to participate in this mechanism. However, its mechanism and physiological consequences for lipid metabolism are not yet known [14]. In addition to these biomarkers, several others have been reported to be associated with inflammatory and infectious conditions, such as serum amyloid A (SAA), C-reactive protein (CRP), IL-1β, IL-6, procalcitonin, and metalloproteinases 8 and 9 [16], and also relative HDL size [17]. As biomarkers of TB infection are still not understood, and plasma cholesterol and lipoproteins are also associated with infectious and inflammatory conditions, this study provides an evaluation of plasma lipoprotein cholesterol, apolipoprotein A-I, SAA and HDL particle size for use as biomarkers of patients with suspected TB.

METHODS

Study design

This is an observational study carried out at Instituto Brasileiro para a Investigação da Tuberculose/Fundação José Silveira (IBIT/FJS), which is a reference center for TB treatment in Salvador, Bahia, Brazil. In total, 129 individuals, who presented with respiratory symptoms, including cough and sputum for more than 2 weeks and no previous TB history, were sequentially enrolled in the study between September 2015 and September 2016. These individuals were submitted to at least two sputum smear microscopies and a rapid molecular test for TB using Xpert MTB/RIF. Lowenstein Jensen medium culture was also performed in all positive patients to confirm the presence of the *M. tuberculosis* complex.

The volunteers were separated into two groups, according to the results of TB diagnosis: a TB group or a non-TB group. Patients with extra-pulmonary TB associated with pulmonary TB were included in the TB group. The presence of exclusive extra-pulmonary TB was excluded from both groups.

The study design inclusion and non-inclusion criteria are shown in the flowchart (Fig. 1).

Blood from all patients was collected into CAT serum Sep Clot Activator and EDTA K2 tubes (Greiner Bio-one) to obtain serum and plasma, respectively, which were preserved at −70 °C to perform further biochemical laboratory tests in a fully automated clinical chemistry analyser.

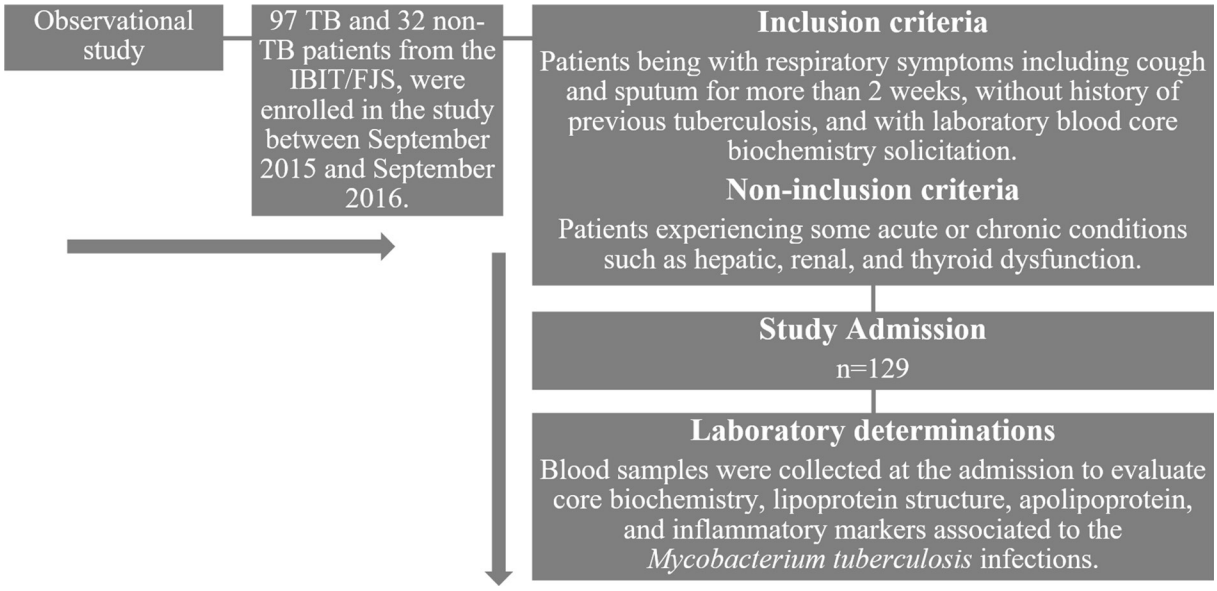

Fig. 1. Flowchart depicting the study design, inclusion and non-inclusion criteria, study admission, and laboratory tests performed.

This study was conducted in cooperation with the Lipid Metabolism Laboratory of the Heart Institute (InCor) of Medical School Hospital, University of São Paulo, Brazil. The study protocol was conducted according to the Declaration of Helsinki and was approved by the Ethical Committee of the Maternidade Climério de Oliveira, at Federal University of Bahia. Written consent was obtained from all individuals after a complete description of the protocol.

Sample size calculation

The sample number used was sufficient to obtain a minimum statistical power of 80% (1−β) and to detect a minimum difference of 10 units of SAA concentration between TB and non-TB patients. The sample calculation was performed on WINPEPI for Windows, version 11.48 (Joe Abramson, PEPI; programs for epidemiologists), and GraphPad StatMate 2.0, Sample size and Power, for Windows.

Biochemical determinations

Serum and plasma samples were obtained after 12–14 h of fasting into a CAT serum Sep Clot Activator and EDTA K2 tubes (Greiner Bio-one). The cholesterol lipid profile and triglyceride values were obtained by enzymatic methods. HDL cholesterol (HDL-C) and LDL cholesterol (LDL-C) were determined by homogeneous direct methods (Wiener-Lab), using the fully automated Konelab 60i, Wiener Clinical Chemistry Analyzer (Wiener-Lab). VLDL cholesterol (VLDL-C) was calculated using the Friedewald formula if triglyceride values were <150 mg dL^{-1}. Apolipoprotein A-I (ROCHE) and SAA (Siemens) were measured using immunoturbidimetric and nephelometric assays, respectively. All biomarker measurements were validated using control samples provided by the National Quality Control Program (PNCQ, Brazil) of the Brazilian Society of Clinical Analyses and by Quality Control for Laboratories (Controllab, Brazil), which had a proficiency test qualification from the Brazilian Society of Clinical Pathology/Laboratory Medicine (SBPC/ML) at the time of the measurements.

TB diagnosis

A diagnosis of TB was made by the following methods. (a) Bacilloscopy: assay for AFB on sputum smear microscopy, performed following standard procedures [18]; (b) Xpert MTB/RIF: this molecular assay was performed according to the manufacturer's recommendations and in line with the Manual of Recommendations for the Control of Tuberculosis in Brazil of the Ministry of Health [19]; (c) mycobacterial culture: *Mycobacterium* culture was performed using two tubes of Lowenstein Jensen medium following standard procedures, and *Mycobacterium* growth detection was observed weekly [18]; and finally (d) *M. tuberculosis* complex identification: this was confirmed by the TB Ag MPT64 BIOEASY identification test (Standard Diagnostic, Republic of Korea) [20].

Table 1. General demographic description of the studied population

Demographic description	
Sex	
Male – *n* (%)	66 (57.4)
Female – *n* (%)	63 (42.6)
AFB identification – *n* (%)	81 (73.6)
Age – years; mean (95% CI of the mean)	42 (39–45)
Weight – kg; mean (95% CI of the mean)	64.0 (60.8–67.2)
N	129 (100%)

AFB, acid-fast bacilli; CI, confidential interval; *n*, absolute frequency.

HDL diameter

Plasma HDL size was determined by light scattering, using the Zetasizer Nano ZS90 Platform (Malvern), after precipitation of lipoprotein-containing apolipoprotein B, by addition to a solution of PEG 8000 (200 g L^{-1}). The supernatant containing HDL was diluted in 0.15 M NaCl containing 0.01% EDTA (pH 7.5). The resulting solution was passed through a 0.22 µm filter. The diameter (nm) of the HDL particles in solution was determined by collecting readings obtained from the light scattering at an angle of 90°, and values were expressed using the average obtained from ten readings (one reading per run) [21].

Statistical analysis

GraphPad InStat v.3.05, GraphPad Prism 8.0.1 and GraphPad SatMate 2.0 software (GraphPad Software) were used for data analysis. Data descriptive analysis (continuous quantitative variables) was performed by calculating the centrality and dispersion estimates to obtain summary measures. Those calculations were followed by a D'Agostino normality test. Before initiating inferential statistical analysis, Grubb's test was performed to detect outliers. The outlier test was followed by parametric and/or non-parametric tests, depending on the data distribution type around the mean. Data analysis was considered significant when the differences obtained showed critical levels (*P*) less than 5% ($P<0.05$) for a 95% confidence interval (CI).

Receiver operating characteristic (ROC) curve analysis was used to calculate sensitivity, specificity, positive and negative predictive values, the area under the curve (AUC), and cutoff as the optimal operating threshold (i.e., optimality means that the sum of sensitivity and specificity was maximal) of lipid markers [22]. The likelihood ratio (LR) and the diagnostic test efficiency (%) were calculated from contingency tables using Fisher's test with Katz approximation. A linear correlation test was also performed.

RESULTS

Table 1 shows the study population's general demographic characteristics.

Fig. 2 shows the lipid profile, apolipoprotein A-I and SAA concentration of individuals from the TB and non-TB groups. Total cholesterol (TC), HDL-C and apolipoprotein A-I were lower in the TB group. SAA concentration was significantly higher in the TB group.

The HDL particle size between the TB (10.16±1.02 nm, $n=55$) and non-TB groups (9.62±0.67 nm, $n=31$) was significantly different (Mann–Whitney U test, $P<0.009$).

Fig. 3 shows Pearson's linear correlation between SAA and HDL-C. SAA and HDL-C for the TB and non-TB groups ($r=-0.26$, $P=0.04$, and $r=-0.34$, $P=0.04$, respectively) were negatively correlated.

Linear correlation analysis among the other evaluated biomarkers between the TB and non-TB groups was not significant (e.g., SAA and apolipoprotein A-I).

By performing Fisher's exact association test, we evaluated the relationship between the presence or absence of TB with SAA, HDL-C and apolipoprotein A-I. In addition, the ROC curve was analyzed to determine the cutoff values for SAA, HDL-C and apolipoprotein A-I. For SAA, the cutoff value was <83.85 mg L^{-1}, with a LR and test efficiency of 23.12 and 84.8%, respectively (Fig. 4a). The cutoff point for HDL-C concentrations was >44.5 mg dL^{-1}, with a LR of 12.95 and test efficiency of 47.33% (Fig. 4b). For apolipoprotein A-I, the cutoff value was >118.5 mg dL^{-1} (Fig. 4c), with a LR of 0.33 and test efficiency of 22%.

Regarding HDL size and TC, the ROC AUC was ≤70% (0.65 and 0.70, respectively). Thus, we did not consider these variables to be of value for TB diagnostic purposes.

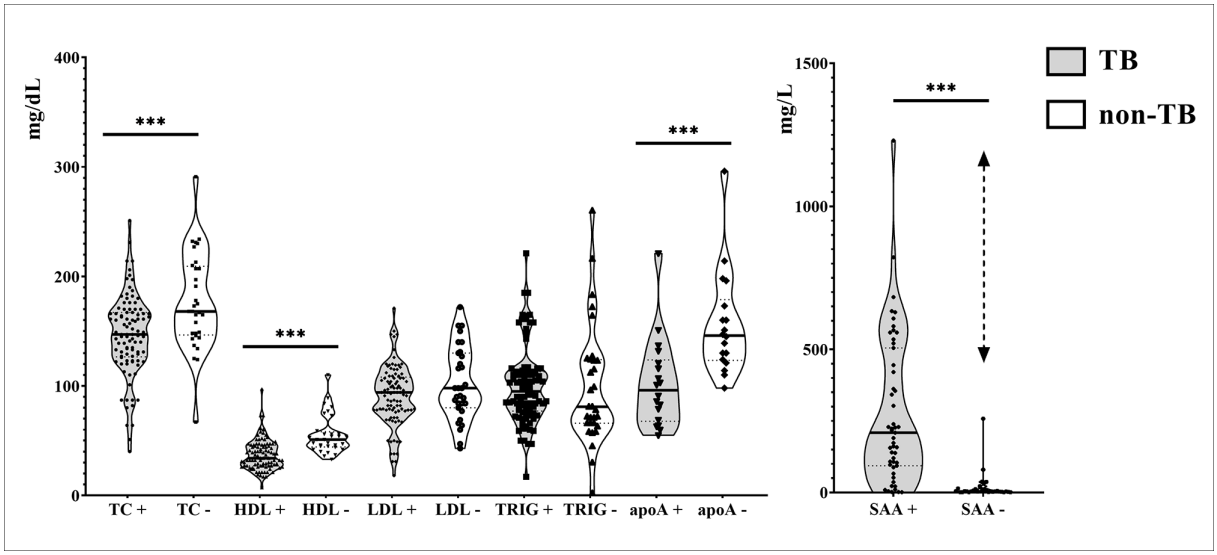

Fig. 2. Comparative analysis of serum biomarker concentrations among individuals with respiratory symptoms with and without laboratory evidence of TB, i.e., positive bacilloscopy (+) (TB group) or negative bacilloscopy (-) (non-TB group). TC, total cholesterol; *n*(+)=97, *n*(-)=32. HDL, HDL-cholesterol; *n*(+)=97, *n*(-)=32. LDL, LDL-cholesterol; *n*(+)=97, *n*(-)=32. TRIG, triglycerides; *n*(+)=97, *n*(-)=32. apoA, apolipoprotein A-I; *n*(+)=18, *n*(-)=18. SAA, serum amyloid A; *n*(+)=51, *n*(-)=32. Mann–Whitney U test, significance was determined at $P<0.05$ for a 95% confidence interval. ***$P<0.0001$.

DISCUSSION

The need to identify new biomarkers for TB diagnosis arises due to difficulties in making a confirmed diagnosis [23]. The current gold standard for TB diagnosis is still sputum culture, which takes weeks until a result is reached, and molecular diagnosis (Xpert MTB/RIF), which can be supported as a globally accessible test [24]. Therefore, the most widely used diagnostic test is the detection of mycobacteria in sputum, with sensitivities that can vary from 34 to 80%. Thus, the identification of endogenous biomarkers or host biomarkers is needed. These biomarkers could be useful to increase sensitivity to pulmonary and extra-pulmonary TB diagnosis, distinguishing TB infection from disease, evaluating active disease risk progression from latent TB, and aiding with TB treatment response.

According to Shu *et al.* [6], early detection is more difficult because the clinical samples are paucibacillary. A negative smear microscopy diagnosis should be given more attention since these patients are responsible for about 17% of TB transmission. Currently, several endogenous and exogenous TB biomarkers are already being studied, suggested to aid diagnosis and treatment. Of these, those biomarkers related to pulmonary TB diagnoses, such as procalcitonin, IL-2, IL-9, IL-10, IL-13 and IL-17, TNF-α

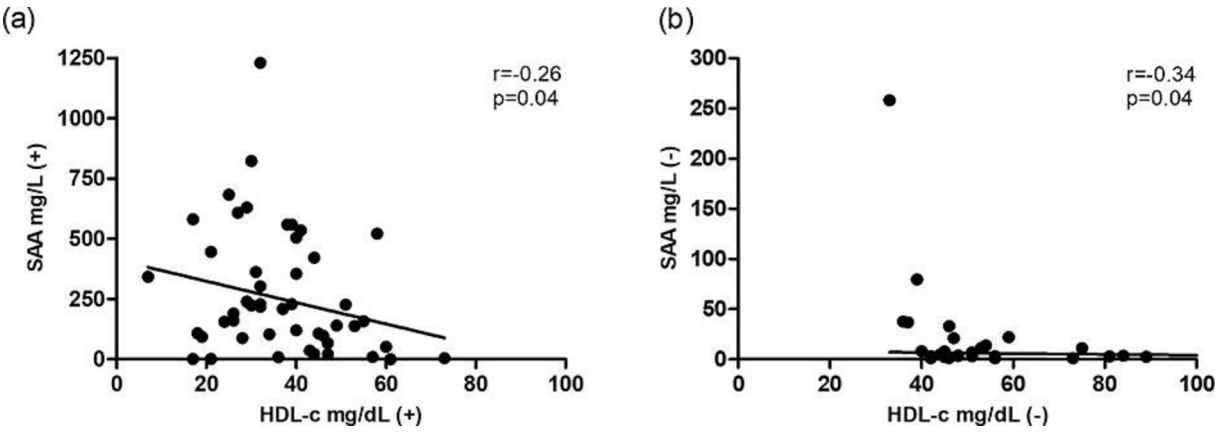

Fig. 3. Correlation between serum amyloid A (SAA) and high-density lipoprotein cholesterol (HDL-C) in patients with positive bacilloscopy (a) (TB) and negative bacilloscopy (b) (non-TB). (+), TB group; (-), non-TB group. Pearson's linear correlation test. Significance was determined at $P<0.05$ for a 95% confidence interval.

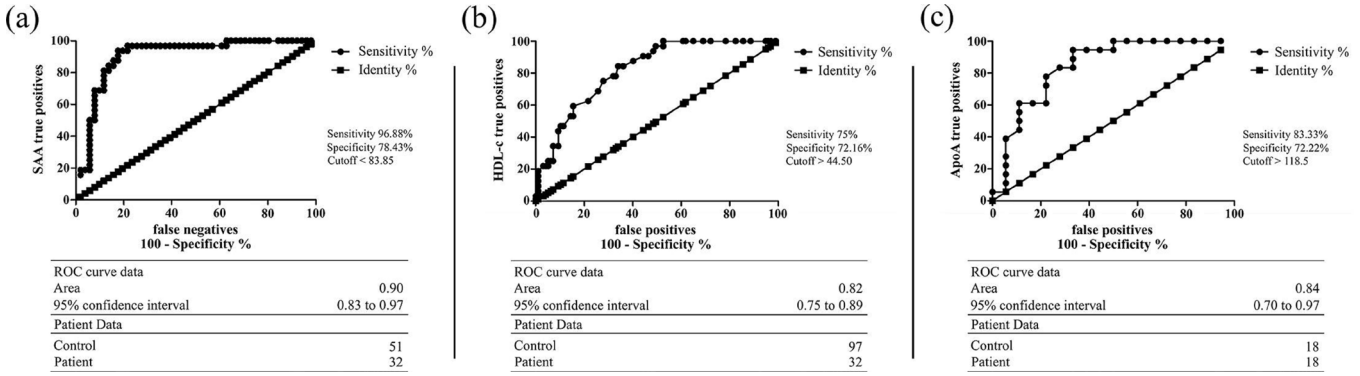

Fig. 4. Receiver operating characteristic (ROC) curves for serum amyloid A (SAA) (a), high-density lipoprotein cholesterol (HDL-C) (b) and apolipoprotein A-I (apoA) (c) from TB patients and non-TB individuals. SAA, HDL-C and apolipoprotein A-I values were imputed as true positives (from TB patients) and false positives (from non-TB individuals).

and CRP, have been extensively reported [16]. However, the greatest difficulty associated with these biomarkers is to distinguish between community-acquired pneumonia and TB patients. In addition to sera, urine and pleural effusion markers, such as lipoarabinomannan, IFN-γ and adenosine deaminase, have been reported [6, 25].

Perez-Guzman *et al.* [26] and Deniz *et al.* [11] reported an association between low TC concentrations and several diseases, including TB. TB infection has been linked to hypocholesterolaemia, and current antimycobacterial therapy could affect this lipid profile [7]. In the present study, symptomatic AFB-positive patients showed lower TC, HDL-C and apolipoprotein A-I levels. Regarding the lipid profile, we observed that the values relate not only to lipoprotein metabolic function [27, 28] but also are structural since the study demonstrated significantly greater HDL particle size in TB patients. These results correlated with significantly higher levels of SAA (i.e. about 12 times more SAA) with HDL particles.

Another notable finding here is an observed reduction of TC, HDL-C and apolipoprotein A-I, followed by increased SAA in individuals with pulmonary symptoms and a negative AFB smear. This finding could be used to help both in differentiating between cases of TB (e.g., pulmonary and extra-pulmonary), in providing information about the risk of development of active disease (i.e., latent TB), as well as supporting the response to TB treatment. Therefore, clinical practice needs to be redirected to evaluate new serum biomarkers before releasing patients with pulmonary symptoms without any treatment for TB. Several studies have focused on new biomarkers [8–12, 26, 29, 30], suggesting the routine use in the health practice of new biomarkers, such as TC and HDL-C, which are already also being suggested for monitoring during antimicrobial evaluation [30]. We have shown a negative correlation between SAA and HDL-C, regardless of whether the individuals were TB bacilli positive or negative, indicating HDL-C reduction in the presence of occluded infection (paucibacillary), as observed by Shu *et al.* [6].

To verify the dependence between the study variables and to establish their cutoff points for future clinical use, ROC curve analysis was performed among individuals with positive TB and cases with a negative AFB smear. Our data are endorsed based on initial findings from the research groups of Deniz [11] and Perez-Guzman [26], who found plasma TC concentration reductions in many diseases, including TB. Our study showed that at least 1% of the cases are contained within the SAA cutoff >83.85 mg L^{-1}, although being classified as negative on bacilloscopy, with a lipid profile (i.e., TC, HDL-C and apo A-I) similar to patients classified as positive for AFB, with a LR of 23.12 and an SAA diagnostic efficiency of 84.84%. As noted, this evidence was given for HDL-C concentration analysis when values were <44.5 mg dL^{-1} in about 1% of the so-called negative smear-stain cases and 29% of cases classified as positive on smear-stain. This analysis showed a positive predictive value of 96% and a LR for HDL-C of 12.95. The same was found for apolipoprotein A-I; 42% (apon A-I <118.5 mg dL^{-1}) of the studied samples were referred to as negative on smear-stain, but at the same apolipoprotein A-I cutoff, 14% of the patients were classified as positive on smear-stain. Although apolipoprotein A-I was shown to have low predictive values, the AUC was 0.84 with both positive and negative cases, which made it possible to be used for diagnostic purposes.

ROC curves were used as the analytical procedure for AUC and cutoff point calculation because it optimizes sensitivity and specificity from a test result, defined as positive or negative [31]. A study carried out in Africa by Chegou and collaborators [32] with 716 participants, 487 classified as lacking evidence of TB, observed seven suggestive biomarkers to support TB diagnosis, such as apolipoprotein A-I, SAA, CRP, IFN-γ, transthyretin, complement factor H and inducible protein 10. These biomarkers helped to confirm the diagnosis of 210 patients, with a mean sensitivity of 93.8% [95% CI; 84.0–98.0%] and specificity of 73.3% (95% CI; 65.2–80, 1%) and positive and negative predictive values of 60.6% (95% CI; 50.3–70.1%) and 96.4% (95% CI 90.5–98.8%), respectively [32].

Regarding the predictive values and AUC for SAA, our results agree with those obtained by Chegou *et al.* [32] for positive and negative TB cases. However, for apolipoprotein A-I values, predictability and AUC were slightly lower and higher, respectively, in our study.

Considering the HDL size and TC values, since the AUCs were ≤70%, these two parameters were not considered here to be useful in support of TB diagnosis. In contrast, some authors consider TC as a serum biomarker for the therapeutic monitoring of TB [7, 11, 26, 33, 34]. Regarding lipoprotein diameter, we have not found studies in the literature for comparison.

Although our study had groups with unequal sizes (i.e., non-TB compared to TB cases), it did not limit us from reaching a pre-established statistical power. Although there are study limitations, our findings regarding TB serum biomarkers presented in this study seem promising.

Serum biomarkers such as HDL-C, apolipoprotein A-I and SAA should be included in the diagnostic evaluation of TB, mainly in symptomatic patients. The present study indicates that these biomarkers, although not specific to *M. tuberculosis* diagnosis, could be evaluated routinely to help TB diagnosis, especially in patients with negative AFB samples.

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Conflicts of interest
The authors declare that there are no conflicts of interest.

Ethical statement
The individuals were included in the study after being informed about the protocol risks and benefits, and signed the consent form. This study was approved by the Research Ethics Committee of the Maternidade Climério de Oliveira of the Federal University of Bahia - CEP/MCO/UFBA, letter no. 2.017.881, CAAE number: 48714115.6.0000.5543, 17 April 2017.

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