ELSEVIER

Archives of Medical Research ■ (2018) ■

Archives of Medical Research

ORIGINAL ARTICLE

Improvement in the Diagnosis of Tuberculosis Combining *Mycobacterium Tuberculosis* Immunodominant Peptides and Serum Host Biomarkers

Juan E. López-Ramos,[a,d] Noe Macías-Segura,[a,f] Betzaida Cuevas-Cordoba,[a] Zaida Araujo-Garcia,[b] Yadira Bastián,[c] Julio E. Castañeda-Delgado,[c] Edgar E. Lara-Ramirez,[a] Benjamin Gándara-Jasso,[a,e] Carmen J. Serrano,[a] Eva Salinas,[d] and Jose A. Enciso-Moreno[a]

[a]*Unidad de Investigación Biomédica de Zacatecas, IMSS, Zacatecas, Zacatecas, México*
[b]*Laboratorio de Inmunología de Enfermedades Infecciosas, Instituto de Biomedicina Dr. Jacinto Convit, Universidad Central de Venezuela, Caracas, Venezuela*
[c]*Catedras CONACYT, Unidad de Investigacion Biomedica de Zacatecas, IMSS, Zacatecas, Zacatecas, México*
[d]*Departamento de Microbiología, Centro de Ciencias Básicas, Universidad Autónoma de Aguascalientes, Aguascalientes, México*
[e]*Departamento de Enseñanza e Investigación, Secretaría de Salud de Zacatecas, Zacatecas, México*
[f]*Departamento de Fisiología y Farmacología, Centro de Ciencias Básicas, Universidad Autónoma de Aguascalientes, Aguascalientes, México*

Received for publication March 3, 2018; accepted July 9, 2018

Background. Pulmonary tuberculosis (PTB) is a public health problem with 10.4 million new cases reported in 2017 (1). According to the World Health Organization (WHO), accurate diagnostic tests based in serum biomarkers to detect new cases of tuberculosis are necessary.

Aim of the study. To evaluate antibodies against *Mycobacterium. tuberculosis* (Mtb) peptides (Ab-Mtb) and three soluble host biomarkers by ELISA serial multiple test in sera from non-infected controls (NIC, $n = 31$), latent tuberculosis (LTB, $n = 37$) and PTB ($n = 28$) patients in a diagnosis tuberculosis assay.

Materials and methods. Levels of four Ab-Mtb peptides derived from Mtb and three host response molecules in serum from NIC, LTB and PTB were evaluated by ELISA as tuberculosis biomarkers. Multiple comparisons tests, determination of diagnostic values and ROC curves were performed. Serial and parallel multiple tests were performed with the biomarkers with the highest discriminatory capacity to improve diagnostic values of the test.

Results. We found significant differences between biomarkers levels in PTB comparing LTB and NIC to all candidate biomarkers; peptides P12033, P12037, and serum biomarkers such as sCD14 and chemokine CXCL9 showed the best sensitivity and specificity, the highest discriminatory power, and the best area under the curve (AUC) individually. In serial multiple tests, P12037 and sCD14 together have 92% of sensitivity and 91% of specificity, with positive and negative likelihood ratios greater than 10.

Conclusions. Ab-Mtb peptide P12037 and sCD14 could be applied in a diagnostic test for suspected PTB to improve accuracy and time to diagnosis and could be implemented in a POCT device which can be affordable.

Key Words: Tuberculosis, Host response biomarkers, Antibody detection, ELISA, Serodiagnosis tests.

Introduction

The World Health Organization (WHO) reported in 2017 that Tuberculosis (TB), mainly caused by *Mycobacterium tuberculosis,* remains as one of the top 10 causes of death worldwide in 2016, provoking more deaths than HIV and

Address reprint requests to: Jose A. Enciso-Moreno, Unidad de Investigación Biomédica de Zacatecas. UIBMZ-IMSS, Interior de la Alameda #45, Col. Centro, Zacatecas, Zacatecas, México; Phone: +524929226019; E-mail: enciso_2000@yahoo.com

0188-4409/$ - see front matter.
https://doi.org/10.1016/j.arcmed.2018.07.003

malaria; this makes Mtb the most lethal infectious pathogen (1). There were an estimated 10.4 million new (incident) TB cases worldwide, of which 65% were men, 29.1% were women and 6.9% were children (1). Also, an estimated of 490,000 people developed multiple drug resistance TB (MDR-TB) worldwide in 2016, and out of these 490,000 people, 240,000 people died (2). MDR-Mtb strains with additional resistance have now been found in every region of the world (3).

Approaches to decreasing TB morbidity, mortality, and Mtb transmission rely on correct and timely diagnosis, effective treatment, and prevention of infection. Up until recently, LTB was thought to represent a uniform state (4). However, it has become clear that LTBI and TB have to be considered as a broad spectrum of states that differ by the degree of pathogen replication, host resistance, and inflammation (4−6), making more difficult to have a TB diagnosis.

Conventional LTB diagnosis relies on the tuberculin skin test (TST) and interferon-gamma release assays (IGRA). Neither TST nor IGRA can discriminate latent infection from active disease. For the diagnosis of PTB, sputum smear microscopy, cultures and, nucleic acid amplification tests (NAAT) are the currently used tools. However, smear microscopy lacks sensitivity and cannot detect paucibacillary cases as the culture can do it, but cultures are expensive, require BSL3 labs, and obtaining results takes a long time, while NAAT are expensive and not easily deployable at the peripheral level. Recently, Pai reviewed the current best diagnostic tools available for TB diagnosis and described the most important gaps and translational challenges for developing innovative tuberculosis tests (7). He suggests a sputum-based replacement test for smear-microscopy and a non-sputum-based biomarker test for all forms of TB.

The translational challenge for several NAAT is to convert them into more affordable assays. For the non-sputum TB test, the biggest challenge is the lack of validated biomarkers. Although considerable efforts are being made to identify biomarkers that can meet some of these needs, progress has been slow (7,8). Thus, the discovery of TB biomarkers is an important goal in current TB research; unfortunately, no such markers are currently available.

In a previous work, using an experimental animal model of PTB (9), we identified a transcriptome of the host response associated with the progression of tuberculosis. In this specific transcriptome, sUPAR, sCD14, and CXCL9 were highly up-regulated in the blood of patients with active tuberculosis (Patent submitted, 2012). Moreover, on another parallel study, we demonstrated the usefulness of an enzyme-linked immunosorbent assay (ELISA) for the detection Ab-Mtb peptides in the serum of patients with PTB and extrapulmonary TB (EPTB) (10). This assay uses 20 amino acid- long, non-overlapped synthetic peptides that spanned Mtb ESAT-6 and Ag85A sequences. The validation cohort included patients with PTB, patients with EPTB, individuals without EPTB, individuals with leprosy and NIC. For the PTB group, two ESAT-6 peptides (12033 and 12034) had the best sensitivity values, while the best specificity values were for an ESAT-6 peptide (12037) and an Ag85A-peptide (29878).

Here, we evaluated the levels of Ab-Mtb peptides of these four different derived peptides from Mtb and we combined the results with the evaluation of three soluble host biomarkers in serial multiple tests in sera from NIC, LTBI and PTB patients.

Materials and Methods

Study Participants

We included 96 adult individuals, recruited between September 2006 and May 2011. Groups were stratified as NIC ($n = 31$), LTB ($n = 37$) and PTB ($n = 28$). The inclusion criteria for the NIC group included TST (<9mm) and QuantiFERON-Gold® (QFN) negative. The LTB group comprised asymptomatic subjects with TST$>$ 10 mm and/or positive to Quantiferon *in tube* IGRA test. The PTB group was confirmed by either culture (positive) and/or AFB (acid-fast bacilli) smears.

Each of the individuals was clinically evaluated by a certified pneumologist and answered a standardized clinical-epidemiological questionnaire. All participants signed an informed consent letter fulfilling all international regulations and requirements of the ethics and the National Research Committee at IMSS (protocols approval: IMSS CNIC 2005 3301-18 and IMSS CNIC 2005 3301-19).

Serum samples and ELISA determinations

Two blood samples without anticoagulant were obtained from each participant. Samples were centrifuged for 5 min at 300 × g to obtain serum aliquots were stored at −20°C until use.

Antibodies levels of Ab-Mtb peptides derived from *Mtb* (P12033, P12034, P12037 and P29878) and three serum host response proteins (sCD14, sCXCL9, sUPAR) were evaluated by ELISA (BioAssays Systems, USA and R&D systems) following manufacturer's instructions. One hundred microliters of each standard and serum samples (diluted and not diluted) were applied separately to each well in duplicates. Plates were read on a microplate reader (Multiskan Ascent 96/384 Plate Reader, MTX Lab Systems, Inc. USA).

The Mtb derived synthetic peptides sequences and ELISA methodology to quantify serum Ab-Mtb peptides were previously described (10). Serum samples were used diluted and the OD values for each well was measured in an ELISA microplate reader as described previously.

Statistical Analysis

The OD values for Ab-Mtb peptides and soluble protein concentrations were evaluated for normality by Kolmogorov-Smirnov or Shapiro-Wilks tests according to the number of subjects per group, and homoscedasticity by Levene's tests. Differences of biomarkers between PTB, LTB and NIC groups were evaluated by Kruskal Wallis tests, and Tamhane or Tukey post-tests performed specific comparisons. Cases in which no differences between LTB and NIC results were found, the groups were considered as a single one group (no-PTB) and results were compared respect to the PTB group through Mann-Whitney *U* tests.

To identify the biomarkers with the highest power of discrimination (when comparing no-PTB vs. PTB) and the best cut−off values, Linear Discriminant Analysis (LDA) and ROC curve analysis comparing AUC were performed. To evaluate the biomarkers, sensitivity, and specificity (validity), predicted values (safety), Likelihood ratios (accuracy) and Youden's index (global index) were calculated for several cut-off points through single and multiple diagnostic tests applied sequentially (serial testing) and simultaneously (parallel testing). Results were considered significant when two-tailed p values were <0.05. All analyzes were carried out using SPSS (PASW statistics 18; Chicago, IL, USA) and Epidat (Epidat 3.1; Galicia, Spain).

Results

Comparisons of Biomarkers Levels and serum Ab-Mtb peptides Among PTB, LTB and NIC Subjects

ELISA test was used to compare the serum levels of biomarkers CXCL9, sCD14, sUPAR, and Ab-Mtb peptides P12033, P12034, P12037 and P29878 in patients with TB respect to LTB and NIC subjects. Serological levels of sUPAR showed no differences among the PTB, LTB and NIC groups ($p = 0.057$). However, serum levels of CXCL9 ($p < 0.001$), sCD14 ($p < 0.001$), and for Ab-Mtb peptides P12033 ($p < 0.001$), P12034 ($p = 0.032$) and P12037 ($p < 0.001$), were significantly different in the PTB group compared to LTB or NIC subjects, but none of them showed differences between the LTB and NIC groups (except for P29878, $p < 0.01$). Thus, for further analysis, we dichotomized the individuals in only two groups: PTB (patients with active tuberculosis) and no-PTB (LTB and NIC). Comparative analysis is shown in Table 1.

Table 1. Comparative analysis of host response biomarkers levels and serum Ab-Mtb peptides between PTB and No-PTB subjects

	Median Ab and biomarkers levels		
	PTB	No-PTB	*p*
P12033[a]	0.62	0.33	<0.001
P12034[a]	0.60	0.45	= 0.01
P12037[a]	0.60	0.19	<0.001
P29878[a]	0.73	0.37	<0.001
sCD14[b]	2.38	1.42	<0.001
CXCL9[c]	733.0	121.3	<0.001
sUPAR[d]	1.68	1.38	= 0.018

Measurements of antibodies and serum Biomarkers were as follows: [a]OD, [b]μg/mL, [c]pg/mL, and [d]ng/mL.
Mann Whitney *U* test was done and, p values <0.05 were considered as significant.

Assessing the Ab-Mtb peptides and Biomarkers Discriminating Ability to Identify PTB Patients

Given all serum Ab-Mtb peptides and biomarkers showed differences between patients with active PTB and LTB and NIC, a LDA analysis was made to identify the best candidate biomarkers for TB diagnosis. The results show that the groups LTB and NIC are clearly separated from PTB (Figure 1).

According to the LDA, Ab-Mtb peptides P12037 (F = 31.541), P12034 (F = 21.986) and the host proteins sCD14 (F = 36.681) and CXCL9 (F = 19.255) have the highest discriminatory power. However, to assess whether the combination of those biomarkers is helpful, we compare the diagnostic ability of each one, as well as the optimal cut-off point by ROC curve analysis and AUC. According to this sCD14 is the best discriminatory biomarker with an AUC of 0.90 ($p = 0.00$), followed by P12037 with 0.88 ($p = 0.00$), CXCL9 with 0.86 ($p = 0.00$) and P12034 with 0.66 ($p = 0.02$) (Figure 2).

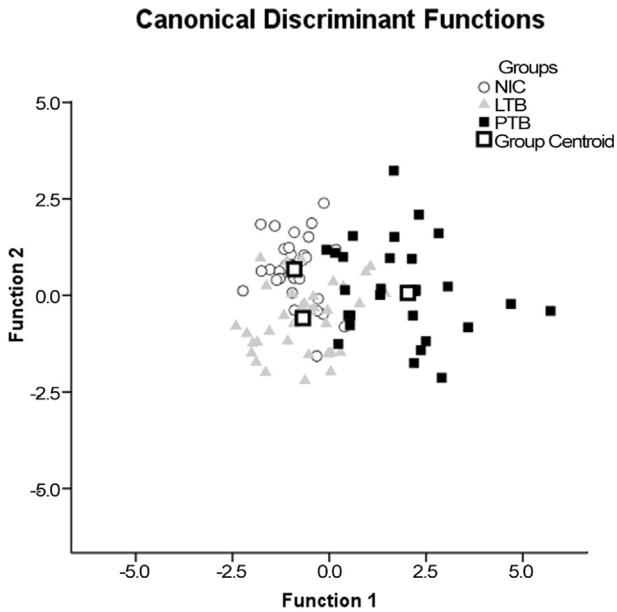

Figure 1. Plot of the individual scores of two canonical functions describing the best Ab-Mtb and Serum Biomarkers to discriminate PTB from No-PTB Patients. NIC in circles; LTB in triangles and PTB in squares are represented in the plot. According to function 1 and function 2 subjects are grouped into 2 main groups: PTB and No-PTB. Centroids (□) indicate the mean discriminant function value of each of the designated groups. The plot was created using SPSS-IBM software (IBM Technology, USA).

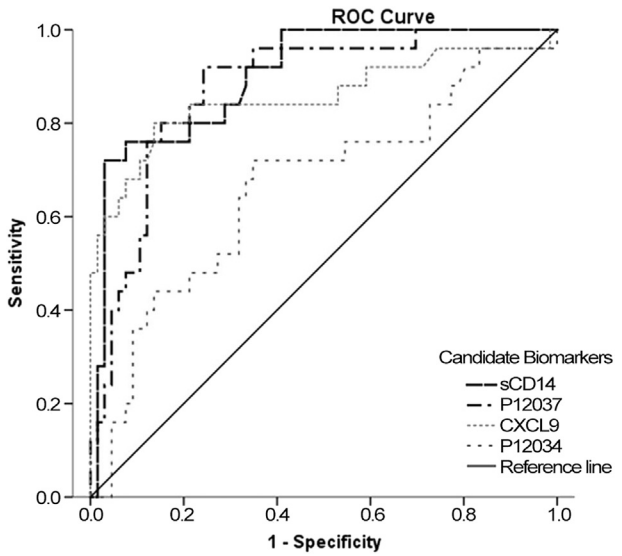

Figure 2. Receiver Operating Characteristic (ROC) for sCD14, P12037, P12034 and CXCL9 for tuberculosis diagnosis. ROC curves determine the different cutoffs according to sensitivity and 1-specificity values; sCD14 and P12037 have the highest AUC. The analysis was done with SPSS-IBM software USA.

Several cut-off points were considered to evaluate the capacity of Ab-Mtb peptides and biomarkers to discriminate subjects with PTB in a single diagnostic test, and one cut-off point was established for each one. The best global measure (Youden's index 0.7) was observed for the Ab-Mtb P1237 at a cut-off point of 0.34 OD with sensitivity of 92.2% and specificity of 76.1%; nevertheless, the highest sensitivity (100%) was obtained with sCD14 at 1.5 µg/mL, although its specificity was low (58.8%); the best specificity (78.8%) was shown by sCXCL9 at 230 pg/mL with a sensitivity of 84%; the cut-off point of 0.51 OD for P12034 showed a sensitivity of 71.4% and a specificity of 63.3%.

A Combination of Biomarkers in Parallel and Serial Tests Increases Their Discriminatory Capacity

Serial and parallel testing was performed for all possible pairs of combinations of these four molecules at the previously considered cut-off point (CD14 & P12037, CD14 & P12034, CD14 & CXCL9, P12037 & P12034, P12037 & CXCL9, P12034 & CXCL9) (Supplementary Table 1).

As expected, parallel tests showed good sensitivity (92−100%) but low specificity (37.9−58.5%). Combination of Ab-Mtb peptides with serum host response biomarkers in serial test offered better specificity values without loss of sensitivity. P12037 and CXCL9 have 95.4% of specificity and 76% sensitivity; P12037 and P12034 have 85.1% of specificity and 67.9% of sensitivity; sCD14 and CXCL9 show 84.9% of specificity and 84% of sensitivity; sCD14 and P12034 have 84.9% of specificity and 72% of sensitivity; P12034 and CXCL9 showed 84% of specificity and 64% sensitivity. The best results were observed in the sCD14 and P12037 combination with 91% of specificity and 92% of sensitivity, as shown in Figure 3.

Regarding accuracy, the likelihood ratios indicate that a positive result is 10 times more likely in people with PTB (likelihood ratio +) and a negative result is 11 times more likely in No-PTB subjects (reason of likelihood −1/ 0.09 = 11.1). Predictive values entail 79% probability to have PTB in a positive result (PPV), and 97% probability to not have PTB with a negative result (NPV). In global terms, the identification of these biomarkers has a diagnostic efficiency of 83% according to Youden's index.

Discussion

It has been broadly recognized that biomarker's molecules associated with immune functions could be important for TB diagnosis. Here we identified and evaluated the diagnostic potential of several Ab-Mtb peptides derived from antigenic immunodominant Mtb antigens and host response tuberculosis serum biomarkers to identify PTB patients.

The essential interdependence and synergy between cell and humoral immunity has been proved by several studies in B cell-deficient mice and SCID mice, using immunization with monoclonal and polyclonal antibodies. Moreover, studies regarding both the protective and the non-protective roles of Ab-Mtb in infection have been reviewed comprehensively for many decades (11), showing the importance of the Ab-Mtb antigens for the TB control (12−14). Tuberculosis affects children, adults and elderly subjects, although the response levels of Ab-Mtb varies among these populations. Even the identification of Ab-Mtb for active tuberculosis is of particular value in childhood, the greater diagnostic challenges, including the lack of serological data in children, the spectrum of clinical manifestations, the age range and the limitations in the context of the immune system maturation, make more difficult an approach to developing a tuberculosis diagnosis assays in children compared adults. Even more, several studies focused on evaluating serum antibody levels against mycobacterial antigens in children show that these antibody levels vary widely in such population, with sensitivities and specificities from 14−84% in several commercial and in-house developed tests (15). Thus, we selected for this study only samples from adults, as to easily standardize a tuberculosis diagnosis test, and extrapolated the results to children in future studies.

To discover novel target proteins for the development of TB serodiagnosis tests, Khan and cols. made proof-of-concept studies for defining antibody profiles in Mtb infection and disease in the macaque model (16). Using a multiplex microbead immunoassay (MMIA), they found good antibody responses associated with latent and active TB with the combination of Mtb antigens ESAT-6, CFP10,

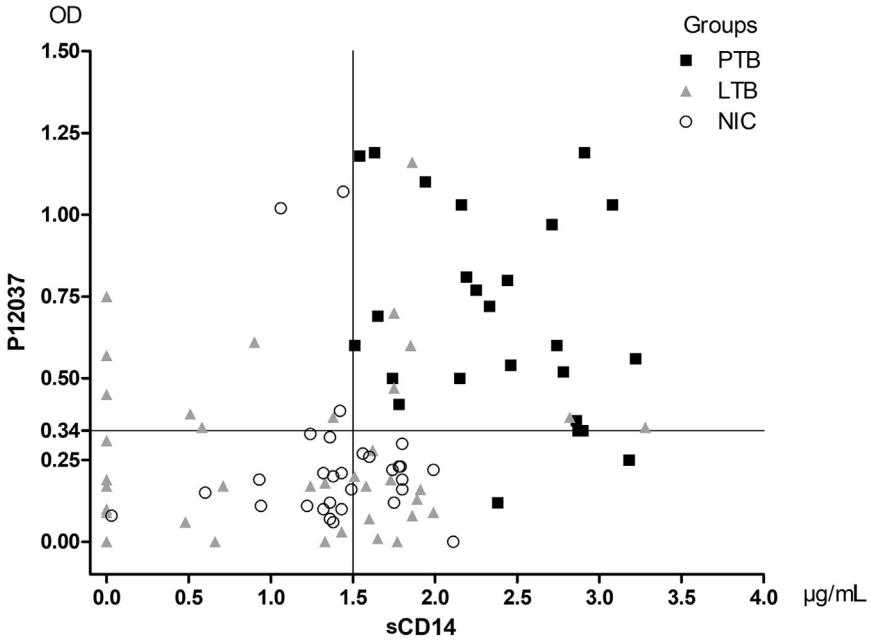

Figure 3. Ab-Mtb P12037 and serum biomarker sCD14 distinguish PTB from No PTB subjects in a serial ELISA testing. Distribution of healthy (NIC-LTB) and diseased (PTB) subjects are separated according to the cut-off values of 0.34 OD for P12037 (y-axis) and 1.5 µg/mL of sCD14 (X-axis). Individuals who have positive values in both tests are considered PTB positive (upper right quadrant), all others are considered as No-PTB.

HSPX, MPT63, MPT53, and Ag85. Besides, the use of whole immunodominant antigens such as ESAT6, CFP10 and Ag85 and their derived peptides as diagnostic tools for humans has been documented previously by our group (10). Peptides such as P12033 and P12034 showed a high sensitivity (96.9, 96.2%, respectively) but low specificity (14.1 and 16.5%,) to detect serum antibodies in TB patients, For this study, sensitivity and specificity were calculated with selected cut-off points, always using the same peptide sequences under the same experimental conditions. To validate the utility of these biomarkers in samples of different population origin, new conditions must be established. Our results confirm that higher levels of serum Ab-Mtb peptides P12033, P12037 and P29878 from ESAT-6, CFP10, and Ag85 antigens, discriminate PTB patients from LTB and NIC subjects in a Mexican population. Nonetheless, their low specificity is still an issue to be used as a diagnostic tool.

The patient's reaction to an infection involves the overexpression of some molecules in serum as a biomarker signature associated with that infectious disease (17–21). Indeed, the elevation of some human host response proteins may work as biomarkers for TB diagnosis. For example, Jacobs and colleagues (22) showed that out of a total of 74 proteins analyzed in plasma samples from African patients with active TB, a biosignature compound of 6 molecules associated to active TB is present. A Luminex multiplex immunoassay using these molecules to detect TB patients showed a sensitivity of 100% (95% CI, 86.3–100%) and specificity of 89.3% (95% CI, 67.6–97.3%). Unfortunately, Luminex assays are costly assays, laborious and require trained personnel making difficult to apply this assay in poor countries where tuberculosis is endemic. Interestingly, none of these molecules were identified in our study. Perhaps, the diverse nature of biomarkers produced in different geographically distributed human populations explain these differences, as well as the fact that Mtb has evolved multiple mechanisms to interfere with the host immune system (23), and has a clonal genetic population structure that is geographically constrained (24).

Sensitivity and specificity are important issues to take into account when using diagnostic tests for tuberculosis as to detect antibody and biomarkers (25); here we used both serial and parallel testing algorithms to improve the sensitivity and specificity in our assays. In the present study, we combined the measurements of serological biomarkers CXCL9, sUPAR, sCD14 and Ab-Mtb peptides P12033, P12034, P12037, and P29878. CXCL9, a cytokine induced by IFN-γ but not by IFN-α/β (26), which is raised as a response to Mtb antigens (27). In our study, CXCL9 discriminated PTB patients from NIC-LTB subjects, with AUC of 0.86. Likewise, sUPAR, whose secretion is stimulated by bacterial endotoxins and cytokines of the innate immune system in monocytes and neutrophils, was able to discriminate PTB patients from LTB and NIC subject. Recently, sUPAR was suggested as a marker for treatment efficiency in pulmonary tuberculosis (28). Moreover, sUPAR expression levels were elevated in PTB compared to NIC, however, sensitivity and specificity were low in our Mexican population compared values of sUPAR in a Creole

Venezuelan population (10); perhaps, the genetic differences in the biomarker expression that these populations may have could explain such differences. Otherwise, sCD14, a 55 kDa glycosylphosphatidylinositol-anchored glycoprotein expressed as soluble protein in serum and on the surface of immune cells such as monocytes, macrophages and polymorphonuclear leukocytes (29), was described as a biomarker for tuberculosis (30) and like we did, they found that sCD14 serum levels could discriminate PTB patients from NIC and LTB, showing its potential as a biomarker in TB diagnosis. Indeed, sCD14 serum levels have the highest capacity to discriminate individuals with PTB respect to NIC, with an AUC of 0.9, and the best values of AUC compared the other identified biomarkers. Even more, our results indicate that simultaneous evaluation of sCD14 and antibody detection Ab-Mtb peptide 12037 show better sensitivity and specificity values compared other assays (30) demonstrating the worth of a combined detection of biomarkers and antibodies in a serial approach. As far as we know, there are few studies describing the combined use of Ab-Mtb and cytokines for tuberculosis diagnosis. For example, Chen and colleagues describe the use of a combined cytokine and Ab-Mtb commercial microarray cytokine testing and house-made colloidal gold nanoparticle kit, reaching sensitivities of 91.03% and specificities of 90.77% (31). Unfortunately, this assay does not follow the WHO recommendations regarding the design of a kit that could be used a point of care sites, given it is complicated to do, quite costly because requires high-level equipment and high levels of skills from operators.

Parallel testing is performed simultaneously to gain in sensitivity, while serial tests are sequential, requiring positive results in the first to perform the second test; this reduces false positives and gain specificity, also minimizes costs since a first negative result avoids carrying out a second test. Our results demonstrate the capacity of multiple diagnostic tests involving the combination of two variables as to increase the accuracy of the analysis, making possible to select the best candidates for PTB patient's discrimination. When we use serial testing and combined the evaluation of 2 different types of serological biomarkers (sCD14 and Ab-Mtb peptide P12037), sensitivity increases up to 92% whereas specificity rose to 91%. We clearly demonstrated that the combined detection of serological biomarkers and antibodies in serum represents a better strategy for diagnostic purposes when they are evaluated simultaneously.

According to the WHO, for TB diagnosis it is necessary (a) a rapid sputum-based replacement test for smear-microscopy; (b) a non-sputum-based biomarker test for all forms of tuberculosis, which should be ideally suitable for use at levels below microscopy centers; (c) a simple, low cost triage test to be used by first-contact care providers as a rule-out test, ideally suitable for use by community health workers; and (d) a rapid drug susceptibility test to be used at the microscopy center level (32). To date, not a single test covers all these requirements. In our study, an ELISA non-sputum-based biomarker test for PTB was developed using the sCD14 biomarker and the P12037 Mtb peptide for the diagnosis of TB. Although the limitations of an ELISA test are linked to the need for laboratory equipment and trained personnel to perform the trial, this proposed serial testing might be a valuable tool with good clinical value due its high sensitivity and specificity values (92% and 91% respectively) as well as likelihood ratio (+10.3 and − 0.09). We consider the next approach is to utilize these two different types of markers to develop a POCT, as the WHO demands the TB diagnosis at the first level of clinical attention. In this sense, a lateral flow assay using these 2 validated biomarkers could be covering most the characteristics that the WHO demands. Thus, based on our results, a quick, simple and low-cost test, ideal to be used in areas at risk of infection with Mtb could be developed; furthermore our ELISA test shows high levels of sensitivity and specificity not found in the conventional TB test, neither the molecular biology test or POC test which have been developed to date now are able to discriminate PTB patients from subjects with LTBI or NIC (33−35). Although this idea results attractive, an extended study with an increased population size that includes different geographical locations will be necessary to validate the usefulness in diagnostics.

Conclusions

Biomarkers that fit with the WHO requirements for a TB diagnostic test were identified. sCD14 and serum antibodies to P12037 have excellent levels of sensitivity and specificity for active TB diagnosis when they are evaluated in a serial diagnostic serum test. These biomarkers show a great potential to be used in a POCT.

Acknowledgments

This work was supported by CONACyT, Mexico, and IMSS, Mexico (Grants 14444 and FIS/IMSS/PROT/1363). ELR was a recipient of a Scholarship from CONACYT, Mexico (389725) and IMSS, Mexico (Mat. 99348716) for his Ph.D. studies.

Conflict of Interest: The authors declare no conflicts of interest.

References

1. Brennan Mike, Cobelens F, Lanfranchi B, et al. An International Roadmap for Tuberculosis Research: towards a world free of tuberculosis. WHO Libr Cat Data; 20111−80.
2. Zignol M, Dara M, Dean AS, et al. Drug-resistant tuberculosis in the WHO European Region: An analysis of surveillance data. Drug Resist Updat 2013;16:108−115.
3. Matteelli A, Roggi A, Carvalho ACC. Extensively drug-resistant tuberculosis: Epidemiology and management. Clin Epidemiol 2014;6:111−118.

4. Barry CE, Boshoff H, Dartois V, et al. The spectrum of latent tuberculosis: rethinking the goals of prophylaxis. Nat Rev Microbiol 2009;7:845−855.
5. Esmail H, Barry CE, Young DB, Wilkinson RJ, et al. The ongoing challenge of latent tuberculosis. Philos Trans R Soc B Biol Sci [Internet] 2014;369:20130437.
6. Dorhoi A, Kaufmann SHE. Pathology and immune reactivity: understanding multidimensionality in pulmonary tuberculosis. Semin Immunopathol 2016;38:153−166.
7. Pai M. Innovations in Tuberculosis Diagnostics: Progress and Translational Challenges. EBioMedicine 2015;2:182−183.
8. Wallis RS, Pai M, Menzies D, et al. Biomarkers and diagnostics for tuberculosis: progress, needs, and translation into practice. Lancet 2010;375:1920−1937.
9. Hernández-Pando R, Orozco H, Sampieri A, et al. Correlation between the kinetics of Th1, Th2 cells and pathology in a murine model of experimental pulmonary tuberculosis. Immunology 1996;89:26−33.
10. Araujo Z, Giampietro F, Bochichio MDLA, et al. Immunologic evaluation and validation of methods using synthetic peptides derived from Mycobacterium tuberculosis for the diagnosis of tuberculosis infection. Mem Inst Oswaldo Cruz 2013;108:131−139.
11. Glatman-Freedman A, Casadevall A. Serum therapy for tuberculosis revisited: Reappraisal of the role of antibody-mediated immunity against Mycobacterium tuberculosis. Clin Microbiol Rev 1998;11:514−532.
12. Imaz MS, Zerbini E. Antibody response to culture filtrate antigens of Mycobacterium tuberculosis during and after treatment of tuberculosis patients. Int J Tuberc Lung Dis 2000;4:562−569.
13. Jackett PS, Bothamley GH, Batra HV, et al. Specificity of antibodies to immunodominant mycobacterial antigens in pulmonary tuberculosis. J Clin Microbiol 1988;26:2313−2318.
14. Uma Devi KR, Ramalingam B, Brennan PJ, et al. Specific and early detection of IgG, IgA and IgM antibodies to Mycobacterium tuberculosis 38kDa antigen in pulmonary tuberculosis. Tuberculosis 2001;81:249−253.
15. Achkar JM, Ziegenbalg A. Antibody responses to mycobacterial antigens in children with tuberculosis: Challenges and potential diagnostic value. Clin Vaccine Immunol 2012;19:1898−1906.
16. Khan IH, Ravindran R, Yee JA, Ziman M, Lewinsohn DM, Gennaro ML, et al. Profiling antibodies to Mycobacterium tuberculosis by multiplex microbead suspension arrays for serodiagnosis of tuberculosis. Clin Vaccine Immunol 2008;15:433−438.
17. Achkar JM, Cortes L, Croteau P, Yanofsky C, Mentinova M, Rajotte I, et al. Host Protein Biomarkers Identify Active Tuberculosis in HIV Uninfected and Co-infected Individuals. EBioMedicine 2015;2:1160−1168.
18. Helb DA, Tetteh KKA, Felgner PL, et al. Novel serologic biomarkers provide accurate estimates of recent Plasmodium falciparum exposure for individuals and communities. Proc Natl Acad Sci 2015;112:E4438−E4447.
19. Caliendo AM, Gilbert DN, Ginocchio CC, et al. Better Tests, Better Care: Improved Diagnostics for Infectious Diseases. Clin Infect Dis 2013;57(suppl 3):S139−S170.
20. Adekambi T, Ibegbu CC, Cagle S, et al. Biomarkers on patient T cells diagnose active tuberculosis and monitor treatment response. J Clin Invest 2015;125:1827−1838.
21. Zhou F, Xu X, Wu S, et al. ORFeome-based identification of biomarkers for serodiagnosis of Mycobacterium tuberculosis latent infection. BMC Infect Dis 2017;17:793.
22. Jacobs R, Malherbe S, Loxton AG, et al. Identification of novel host biomarkers in plasma as candidates for the immunodiagnosis of tuberculosis disease and monitoring of tuberculosis treatment response. Oncotarget 2016;7:57581−57592.
23. Flynn JAL, Chan J. What's good for the host is good for the bug. Trends Microbiol 2005;13:98−102.
24. Gagneux S, Small PM. Global phylogeography of Mycobacterium tuberculosis and Implications for tuberculosis product development. Lancet Infect Dis 2007;7:328−337.
25. Serrano CJ, Cuevas-Córdoba B, Macías-Segura N, et al. Transcriptional profiles discriminate patients with pulmonary tuberculosis from non-tuberculous individuals depending on the presence of non-insulin diabetes mellitus. Clin Immunol 2016;162:107−117.
26. Farber JM. Mig and IP-10: CXC chemokines that target lymphocytes. J Leukoc Biol 1997;61:246−257.
27. Domingo-Gonzalez R, Prince O, Cooper A, et al. Cytokines and Chemokines in Mycobacterium tuberculosis Infection. Microbiol Spectr 2016;4.
28. Indumati V, Vijay V, Krishnaswamy D, et al. Serum levels of soluble urokinase plasminogen activator receptor (suPAR) as a marker of tuberculosis treatment efficacy. Indian J Tuberc 2017;64:206−211.
29. Wright SD, Ramos RA, Tobias PS, et al. CD14, a receptor for complexes of lipopolysaccharide (LPS) and LPS binding protein. Science 1990;249:1431−1433.
30. Zambuzi FA, Cardoso-Silva PM, Espindola MS, et al. Identification of promising plasma immune biomarkers to differentiate active pulmonary tuberculosis. Cytokine 2016;88:99−107.
31. Chen T, Lin J, Wang W, Fleming J, et al. Cytokine and antibody based diagnostic algorithms for sputum culture-positive pulmonary tuberculosis. PLoS One 2015;10:1−16.
32. Denkinger C, Kik S, Casenghi M. High-priority target product profiles for new tuberculosis diagnostics. Consens Meet high-priority target Prod profiles new Tuberc diagnostics 2014.
33. Dheda K, Ruhwald M, Theron G, et al. Point-of-care diagnosis of tuberculosis: Past, present and future. Respirology 2013;18:217−232.
34. Castan P, De Pablo A, Fernández-Romero N, et al. Point-of-care system for detection of Mycobacterium tuberculosis and rifampin resistance in sputum samples. J Clin Microbiol 2014;52:502−507.
35. Evans D, Papadimitriou KI, Greathead L, et al. An Assay System for Point-of-Care Diagnosis of Tuberculosis using Commercially Manufactured PCB Technology. Sci Rep 2017;7:1−10.

Supplementary Table 1. Analysis of validity (sensitivity and specificity), clinical security (predictive values), likely to outcome in presence or absence of disease (likelihood ratios), and overall evaluation (Youden index). All analysis was performed by multiple diagnostic tests in series and parallel, of the six possible combinations of biomarkers. On the right are shown scatter diagrams of subjects with PTB, LTB and NIC

	sCD14 (1.5 μg/mL) & P12037 (0.34 OD)				**sCD14 (1.5 μg/mL) & P12034 (0.51 OD)**			
	Serial		**Parallel**		**Serial**		**Parallel**	
	Value	**CI 95%**	**Value**	**CI 95%**	**Value**	**CI 95%**	**Value**	**CI 95%**
Sensitivity (%)	92.0	79.4−100	100.0	98.0−100	72.0	52.4−91.6	100.0	98.0−100.0
Specificity (%)	91.0	83.5−98.6	44.8	32.1−57.4	84.9	75.4−94.3	37.9	25.4−50.3
Positive predictive value (%)	79.3	62.8 -95.8	40.3	27.3−53.3	64.3	44.8−83.8	37.9	25.4−50.3
Negative predictive value (%)	96.8	91.7−100	100.0	98.3−100	88.9	80.3−97.4	100.0	98.0−100.0
Likelihood ratio+	10.3	4.8−22.2	1.80	1.5−2.3	4.8	2.6−8.8	1.6	1.3−1.9
Likelihood ratio −	0.1	0.02−0.33	-	-	0.33	0.17−0.62	-	-
Youden index	0.83	0.70−0.96	0.45	0.33−0.57	0.57	0.37−0.76	0.38	0.26−0.50
	sCD14 (1.5 μg/mL) & CXCL9 (230 pg/mL)				**P12037 (0.34 OD) & P12034 (0.51 OD)**			
	Serial		**Parallel**		**Serial**		**Parallel**	
	Value	**CI 95%**	**Value**	**CI 95%**	**Value**	**CI 95%**	**Value**	**CI 95%**
Sensitivity (%)	84.0	67.6−100.0	100.0	98.0−100.0	67.9	48.8−86.9	96.4	87.8−100.0
Specificity (%)	84.9	75.4−94.3	50.0	37.2−62.8	85.1	75.8−94.4	53.7	41.1−66.4
Positive predictive value (%)	67.7	49.7−85.8	43.1	29.5−56.7	65.5	46.5−84.5	46.6	32.9−60.3
Negative predictive value (%)	93.3	86.2−100.0	100.0	98.5−100.0	86.4	77.3−95.4	97.3	90.7−100.0
Likelihood ratio+	5.5	3.1−10.1	2.0	1.6−2.6	4.6	2.4−8.5	2.1	1.6−2.7
Likelihood ratio −	0.19	0.08−0.47	-	-	0.38	0.22−0.65	0.07	0.01−0.46
Youden Index	0.69	0.52−0.86	0.5	0.38−0.62	0.53	0.34−0.72	0.5	0.36−0.64
	P12037 (0.34 OD) & CXCL9 (230 pg/mL)				**P12034 (0.51 OD) & CXCL9 (230 pg/mL)**			
	Serial		**Parallel**		**Serial**		**Parallel**	
	CI 95%	**Value**	**CI 95%**	**Value**	**CI 95%**	**Value**	**Value**	**CI 95%**
Sensitivity (%)	76.0	57.3−94.7	100.0	98.0−100.0	64.0	43.2−84.8	92.0	79.4−100.0
Specificity (%)	95.4	89.5−100.0	58.5	45.7−71.2	84.4	74.7−94.1	57.8	44.9−70.7
Positive predictive value (%)	86.4	69.8−100.0	48.1	33.5−62.6	61.5	40.9−82.2	46.0	31.2−60.8
Negative predictive value (%)	91.2	83.7−98.7	100.0	98.7−100.0	85.7	76.3−95.2	94.9	86.7−100.0
Likelihood ratio+	16.5	5.34−50.8	2.4	1.8−3.2	4.1	2.2−7.8	2.2	1.6−3.0
Likelihood ratio −	0.25	0.12−0.51	-	-	0.43	0.25−0.73	0.14	0.04−0.53
Youden index	0.71	0.54−0.89	0.58	0.46−0.70	0.48	0.28−0.69	0.5	0.34−0.66