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Original Article

Diagnostic accuracy of combinations of serological biomarkers for identifying clinical tuberculosis

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Abstract

Introduction: Confirmation of tuberculosis (TB) cases in endemic TB settings is a challenge; obtaining fast and cheap, though accurate, diagnostic tools such as biomarkers is thus urgently needed to enable the early detection of TB. This paper evaluates the diagnostic accuracy of combinations of host serological biomarkers for identifying TB.

Methodology: Enzyme-linked immunosorbent assays (ELISA) were used on 70 Venezuelan Creole individuals for evaluating host biomarkers (i.e. CXCL9, sCD14, MMP9 and uPAR proteins) and anti-synthetic peptides covering certain *Mycobacterium tuberculosis* (*Mtb*) ESAT-6 (P-12033, P-12034 and P-12037) and Ag85A (P-29878) antigen sequences. The target population consisted of adults having active TB (ATB, n = 28), the tuberculin skin test positive (TST^+) or individuals with latent TB infection (LTB, n = 28) and TST^- or control subjects (CTRL, n = 14).

Results: Receiver operator curve (ROC) analysis revealed good biosignature discriminative ability for 5 serological biomarkers; the accuracy of 3 combinations had a good discriminative ability for diagnosing TB. Anti-P-12034/uPAR detected TB with 96.7% sensitivity and 86.0% specificity, followed by anti-P-12033/uPAR having 96.7% sensitivity and 81.4% specificity. Anti-P-29878/MMP9 had the highest sensitivity (100%), but low specificity (52.17%). Biomarker combinations did not prove efficacious for identifying incipient subclinical TST^+TB^- subjects at high-risk for TB.

Conclusions: The anti-P-12034/uPAR combination could be useful for identifying clinical TB patients. Such an approach holds promise for further validation.

Key words: Serological biomarker; synthetic peptide; IgG reactivity; TB.

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Introduction

Tuberculosis (TB) remains a leading cause of morbidity and mortality worldwide, involving 10.4 million cases and 1.4 million deaths annually [1]. One third of the world's population is latently infected with *Mycobacterium tuberculosis* (*Mtb*) and up to 10% of infected individuals develop active TB in their lifetime. Among major TB control challenges is the implementation of sensitive methods for detecting active TB (ATB) and latent tuberculosis (LTB). A vast majority of TB cases occur in developing countries having limited resources, where rapid, inexpensive diagnostic tests would aid limiting the spread of community-acquired infection. Molecular methods for TB diagnosis based on nucleic acid amplification are rapid, highly specific and more sensitive than microscopic examination of smears but less sensitive than culture in smear-negative cases [2]. Microscopy-based TB diagnosis inadequacies have been exacerbated since poor cellular response in infected patients with immunosuppression results in diminished cavity formation and, consequently, in greater proportion of both smear-negative TB and extrapulmonary TB [2,3]. Investment has led to some

progress in developing new diagnostic methods, although the existing pipeline is limited regarding tests for sputum-smear-negative cases, childhood tuberculosis and accurately predicting latent TB reactivation [2,3]. Thus, the need for biomarkers in TB is most crucial in three areas: people having active TB, for predicting durable (non-relapsing) treatment success, patients having latent TB (LTB), to indicate reactivation risk and predict treatment success; and people other than those having active disease, to indicate protection from TB by new vaccines [4].

Host tuberculosis biomarkers can provide prognostic information about potential outcomes for individual patients or for cohort studies. Efforts to exploit antibodies as biomarkers for diagnosing TB have been unsuccessful for decades; however, promising antigens or antigen sequences have been identified [5-7]. Authors' labs have used screening of 2-D fractionated *Mtb* culture-filtrate protein immunoblots, *Mtb* cytosolic and culture-filtrate protein microarrays and *Mtb* DNA expression libraries with sera from TB patients and *Mtb*-infected animals during different TB stages to identify several immunodominant antigens [8,9]. Some immunodominant antigens (e.g. the 38 kDa PhoS protein) are specific for cavitary TB. Others, such as the 81-kDa malate synthase (MS) (Rv1837c) and the 29-kDa MPT51 (Rv3803c), elicit antibodies in patients at different stages of clinical TB (smear-negative or positive TB, non-cavitary, or cavitary TB) and in different types of TB patients (such as $HIV^{-}TB^{+}$ or $HIV^{+}TB^{+}$) [8]. Previous work has shown the usefulness of enzyme-linked immunosorbent assays (ELISA) for serodiagnosis of pulmonary and extrapulmonary TB using synthetic peptides, covering the complete sequence of *Mtb ESAT-6* and Ag85A antigens [7]. That study involved 1,102 individuals (as validation set); the results showed that up to 99.5% sensitivity was achieved in pulmonary TB (PTB) patients and 100% in extra-pulmonary TB (EPTB) patients for the peptide combinations P-12033/P-12034 and P-11005/ P-11006. Since these antibodies were not detected in sera from healthy controls (HC), individuals with non-tuberculous pleural effusion (NEPTB) or individuals suffering leprosy (LP), these results suggested that these combinations proved to be efficacious for identifying clinical PTB and EPTB cases [7].

Biomarkers can also help to advance basic knowledge regarding disease pathogenesis; the need for biomarkers in TB is most crucial in the three above mentioned areas: ATB patients for predicting durable (non-relapsing) treatment success, LTB to indicate reactivation risk and predict treatment success, and people other than those having active disease, to indicate protection from TB by new vaccines [4,10]. A study has reported that MIG/CXCL9 and several of the biomarkers (including IP-10, MCP-1, MCP-2, IL-1RA and MIP-1b) are expressed at many fold higher levels in association with active *Mtb* [11]; MIG/CXCL9 is mainly expressed by monocytes and macrophages; MIG/CXCL9 is strongly induced by IFN-γ, but not IFN-α/β or other T-cell cytokines involved in IP-10 release. TNF-α can induce MIG/CXCL9 alone, but does synergize with IFN-γ [12]. A recent *ex vivo* transcriptional immune biomarker study has highlighted 12 biomarkers being consistently associated with either of the following clinical groups: "upstream" towards culture-positive TB on the TB disease spectrum (CD14, FCGR1A, FPR1, MMP9, RAB24, SEC14L1 and TIMP2) or "downstream" towards a decreased likelihood of TB disease (BLR1, CD3E, CD8A, IL7R and TGFBR2) suggesting correlation with *M. tuberculosis*-related pathology [13].Other authors have identified a seven-marker host serum biosignature (C reactive protein, transthyretin, IFN-γ, complement factor H, apolipoprotein-A1, inducible protein 10 and serum amyloid A) for TB diagnosis, regardless of HIV infection status or ethnicity in Africa. These results hold promise for the development of a field-friendly, point-of-care, screening test for pulmonary TB [14]. Additional studies involving transcriptomic approaches for identifying genes or gene signatures have also demonstrated the presence of biomarkers such as MIG/CXCL9, CD14, MMP9 and uPAR which could be characteristic for latent or active disease [15,16]. Host immunological biomarker-based tests, maybe useful especially if these are based on easily available samples such as serum. We thus investigated host biomarker (CXCL9, sCD14, MMP9 and uPAR) combinations and antibodies directed towards synthetic peptides derived from *Mtb ESAT-6* and Ag85A antigens in serum samples from individuals having active pulmonary TB as tools for TB diagnosis. The present study's second goal was to compare the prevalence of these biomarkers in LTB subjects at risk of developing active TB.

Methodology

Study population and study site

Seventy (37 male and 33 female) Creole subjects were prospectively recruited at the Institute of Biomedicine-Central University of Venezuela "Dr.

Jacinto Convit" in Caracas (the capital of Venezuela); 28 individuals had active pulmonary TB (ATB), 28 were tuberculin skin test $(TST)^+$ or had latent TB infection (LTB), and 14 were TST^- or controls (CTRL).

Clinical features, microscopy, the tuberculin skin test and chest radiograph

Individuals having evidence of clinical symptoms suggesting pulmonary TB infection were diagnosed as having pulmonary TB using at least one of the following previously applied criteria: X-ray suggestive of TB and positive sputum smear or positive sputum culture. Clinical features consistent with TB, such as recent weight loss or inadequate progress of weight gain, prolonged febrile syndrome, night sweats, coughing or wheezing for more than two weeks, were also taken into account [7,17].

Regarding confirmatory TB diagnosis, sputum for investigating alcohol/acid-fast bacilli was collected from all individuals having respiratory symptoms. The investigation was performed by the Laboratory of Tuberculosis at the Institute of Biomedicine-Central University of Venezuela. The smears were stained using the Ziehl-Neelsen direct method. For each sputum sample, 2 tubes of modified Ogawa egg medium and Lowënstein-Jensen were inoculated using the method of Kudoh and Kudoh [18]; in cases of smears with acid-fast bacilli that were not stainable, but isolated from sputum by culture, confirmed TB was defined as *Mtb* isolation in culture. HIV infection status was ruled out by blood tests for all individuals having clinical or paraclinical evidence suggesting pulmonary TB infection.

The tuberculin skin test (TST) was administered according to the Mantoux method; 2 tuberculin units (0.1 mL) of purified protein derivate (RT23 PPD; Statens Serum Institute, Copenhagen, Denmark) were injected intra-dermally, as previously described [17]. Trained professionals did the reading between 48 and 72 hours after administration. Positive test reactivity criteria were based on transversal diameter measurements of the indurations on the volar surface of the forearm (≥ 10mm), according to international guidelines [19].

Standard antero-posterior and lateral chest X-rays (CXRs) were taken from all individuals for TB confirmation; the radiological study was aimed at searching for CXR characteristics regarding lesions suggestive of ATB (Vargas Hospital radiology service in Caracas). Patients having HIV coinfection were excluded from the study.

Treatment was initiated in all identified TB cases where microbiological evidence suggestive of TB and bacteriological confirmation by bacilloscopy or culture were found, and in all cases in which findings of CXR examinations were consistent with clinical symptoms suggesting pulmonary TB infection, as recommended by the Venezuelan National TB Control Programme.

Inclusion criteria for healthy Creoles (the control group) consisted of the absence of a clinical picture suggesting pulmonary TB infection, in which HIV and active TB had been ruled out by blood tests, microbiological assays and CXRs. Individuals who had been prescribed immunosuppressive drugs (i.e., corticosteroids, azathioprine, and cyclophosphamide) were also excluded, as were participants who did not sign an informed consent agreement.

Serological biomarker tests

Blood samples were processed soon after being obtained. Briefly, sera were separated by spinning. ELISA was then used for measuring serological biomarkers CXCL9 (Human CXCL9/MIG Immunoassay, R&D Systems, Minneapolis, USA), MMP9 (Human MMP-9 Immunoassay, R&D Systems, Minneapolis, USA), serum solubleCD14 (sCD14) (Human sCD14 Immunoassay, R&D Systems, Minneapolis, USA) and uPAR (Urokinase Plasminogen Activator Receptor, Human ELISA Kit, BioAssay (uPAR), US Biological, Swampscott, USA) in serum samples according to the manufacturer's instructions.

Synthetic peptides

Four synthetic peptides were selected in accordance with a previous study showing their usefulness for detecting pulmonary TB [7]. Three ESAT-6 and 1 Ag85A antigen sequences were synthesized and used as single peptides (Fundación Instituto de Inmunología de Colombia (FIDIC), Bogotá, Colombia): ESAT-6-peptides P1 (12033), (1-20) MTEQQWNFAGIEAAASAIQG, P2 (12034), (21-40) NVTSIHSLLDEGKQSLTKLA, P5 (12037), (76-95) ISEAGQAMASTEGNVTGMFA and Ag85A peptide P1 (29878), (1-22) MQLVDRVRGAVTGMSRRLVVGAY. Peptides were synthesized by the solid-phase multiple peptide system, based on the *M. tuberculosis* ESAT-6 and Ag85A amino acid (aa) sequence [20-22].

Serological assays using peptides

IgG levels against ESAT-6 and Ag85A peptides were determined in serum by ELISA, as previously reported [7]. Briefly, sera were isolated from venous

blood obtained from controls and patients. 96-wellmicrotitre plates (Immunolon, Birmingham, UK) were coated with 1μg/well of antigen (i.e. ESAT-6 or Ag85A synthetic peptides). Immunoenzymatic assays were performed and standardized in our laboratory for measuring IgG antibodies against peptides. Standardized serum sample dilution was 1:200; following incubation, plates were incubated with peroxidase-conjugated monoclonal anti-IgG antibody (Promega Corporation, Madison, USA). Plates were then washed four times and substrate solution (citrate buffer pH 5.0, H_2O_2 30% and 10 mg ortho-phenylenediaminedihydrochloride (OPD, Sigma-Aldrich, St. Louis, USA) was added and plates were incubated for 15 minutes at room temperature. Color development was measured with an ELISA microplate reader at 492 nm.

Statistical analysis

Receiver operating characteristics (ROC) curves were constructed to assess the methods' overall diagnostic information by comparing the areas under the curve (AUC), obtaining cut-off values, sensitivity, specificity, positive and negative predictive values and likelihood ratio values. Student's t-test was used for comparing the average age between groups and Fisher's exact test for assessing the significance of the differences between percentages of TST positive and negative individuals. Mann U Whitney and Kruskal-Wallis non-parametric tests were used for comparing isotype reactivity differences between groups. A scattergram was plotted using GraphPad Prism software version 5.02 (Trial version, GraphPad Sofware, Inc. San Diego, USA). Statistically significant differences were those having a ≤ 0.05 *p*-value.

Ethical considerations

The present study complied with the Helsinki Declaration. This study was approved by the Institute of Biomedicine-Central University of Venezuela Research Ethics Committee (protocol number FONACIT-2013002319/2013). All participating individuals signed voluntary informed consent forms.

Results

Results concerning age are shown as values of the mean ± standard deviation (X ± SD). Average age was 40.12 ± 13 for ATB, 40.0 ± 12.91 for LTB and 37.79 ± 13.44 for CTRL groups; there were no statistically significant differences regarding average age (Table 1). There were no statistically significant differences concerning gender distribution between males (18/28) and females (10/28) in the ATB group, or males (15/28) and females (13/28) in the LTB group, or males (8/14) and females (6/14) in the CTRL group. Table 1 lists participants' demographic and clinical information.

Table 1 also provides information regarding TST infection status and bacteriological results. TST test reactivity was used for studying delayed-type hypersensitivity (DTH); ≥ 10 mm reactions were considered positive. Regarding the percentage of TST^+ patients, a statistically significant difference was found for the ATB (78.6%) group compared to the LTB (100.0%) and CTRL (0.0%) groups, $p < 0.0001$, (Table 1).Sensitivity was 64.0% for smear and 100.0% for culture in ATB patients, while bacteria were not detected in LTB or CTRL individuals (Table 1). Concerning $smear^+$ status, a statistically significant difference was found between the ATB group (64.0%) and the LTB (0%) and CTRL (0%) groups, $p < 0.0001$, as well as for $culture^+$ status between the ATB group (100.0%) and LTB (0%) and CTRL (0%) groups, $p < 0.0001$, (Table 1). Patients and control individuals < 20 years of age were highly likely to have received one BCG vaccination during childhood as part ofthe increasingly effective Venezuelan National BCG Vaccination Programme (data not shown).

Figure 1 shows optical density (OD_{492}) distribution for the four best IgG anti-peptides. The IgG reactivity against peptides is shown as values of the mean ± standard deviation (X ± SD): P-29878 0.339 ± 0.147 for ATB, 0.317 ± 0.139 for LTB and 0.183 ± 0.068 for

Table 1. Age and gender characteristics, immunological and bacteriological markers.

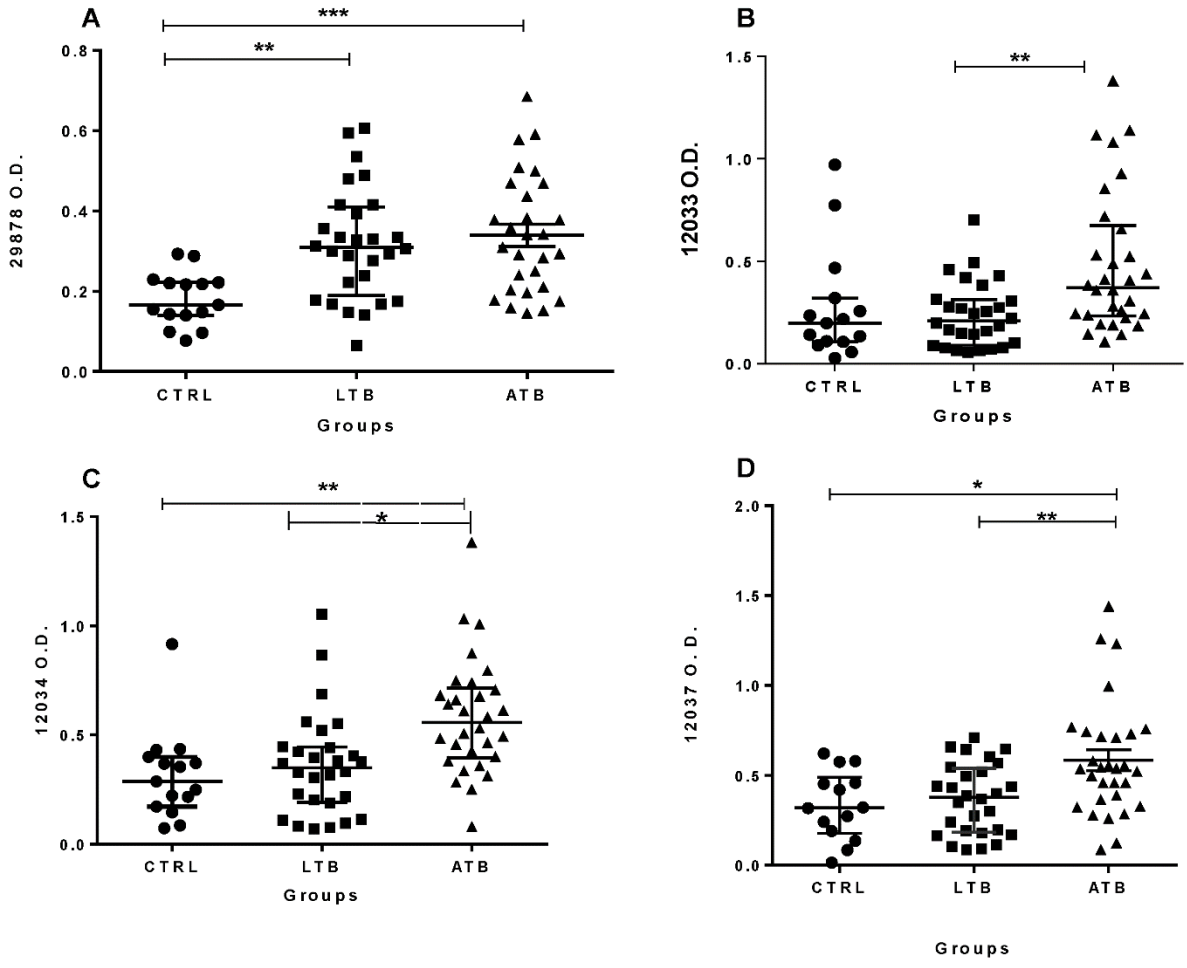
Marker	ATB	LTB	CTRL
Age	40.12 ± 13.46	40.0 ± 12.91	37.79 ± 13.44
Female (%)	35.71	53.57	57.14
Male (%)	64.28	46.42	42.85
TST+ (%)	78.6[(a)]	100.0[(b)]	0.0[(c)]
Smear+ (%)	64.0[(d)]	0.0[(e)]	0.0[(f)]
Culture+ (%)	100.0[(g)]	0.0[(h)]	0.0[(i)]

Age results are shown as the mean ± SD. Subjects having active pulmonary TB (ATB) n = 28, latent TB infection (LTB) n = 28 and healthy controls (CTRL) n = 14. There were statistically significant differences concerning TST+, smear+ and culture+ status between [(a)] versus [(c)] and [(b)] versus [(c)] ($p < 0.0001$), [(d)] versus [(e)] and [(f)] ($p < 0.0001$), and [(g)] versus [(h)] and [(i)] ($p < 0.0001$).

CTRL. P-29878 peptide IgG reactivity was significantly higher in the ATB and LTB groups than in the CTRL group ($p < 0.0005$ and $p < 0.001$, respectively), (Figure 1A), anti-P-12033 readings were 0.440 ± 0.314 for ATB, 0.237 ± 0.159 for LTB and 0.285 ± 0.276 for CTRL groups. P-12033 IgG reactivity was significantly higher in the ATB group than in the LTB group, $p < 0.003$ (Figure 1B); anti-P-12034 readings were 0.564 ± 0.261 for ATB, 0.362 ± 0.236 for LTB and 0.331 ± 0.202 for CTRL. P-12034 IgG reactivity was significantly higher in the ATB than in the LTB ($p < 0.01$) and CTRL ($p < 0.005$) groups (Figure 1C). Anti-P-12037 results were 0.546 ± 0.282 for ATB, 0.368 ± 0.196 for LTB and 0.334 ± 0.191 for CTRL. P-12037 IgG reactivity was significantly higher in the ATB group than in the LTB and CTRL groups ($p < 0.008$ and $p < 0.01$, respectively) (Figure 1D).

Regarding biomarker concentrations, MIG/CXCL9 values were; 468.16 ± 548.66 pg/mL for ATB, 410.98 ± 216.62 pg/mL for LTB and 197.88 ± 55.84 pg/mL for CTRL (Figure 2A). Significantly higher concentrations were observed in the LTB group than in the CTRL group, $p < 0.009$ (Figure 2A). MMP9 biomarker concentrations were; 993.79 ± 670.87 ng/mL for ATB, 373.99 ± 419.15 ng/mL for LTB and 264.59 ± 296.13ng/mL for CTRL. The ATB group had significantly higher MMP9 concentrations than in the LTB ($p < 0.008$) and CTRL ($p < 0.004$) groups, (Figure 2B). The sCD14 biomarker concentrations detected were 378,717.5 ± 925,201.2 pg/mL for ATB, 497,444.0

Figure 1. IgG reactivity test against peptides P-29878(1A), P-12033(1B), P-12034(1C) and P-12037(1D). Distribution of optical density values concerning antibody reactivity among groups: ATB; active pulmonary TB patients (▲), LTB; subjects TST^+ or with latent infection (■) and CTRL; subjects TST^- (●). (1A): ** and *** represent significance of differences in the CTRL group compared with the LTB ($p<$ 0.009) and ATB ($p<$ 0.0009) groups for P-29878. (1B): ** represents the significance between the ATB and LTB ($p<$ 0.003) groups for P-12033. (1C): ** and * represent the significance of differences in the ATB group compared with the CTRL ($p<$ 0.005) and LTB ($p<$ 0.01) groups for P-12034. (1D): * and ** represent the significance of differences in the ATB group compared with the LTB and CTRL ($p<$ 0.005) groups for P-12037.

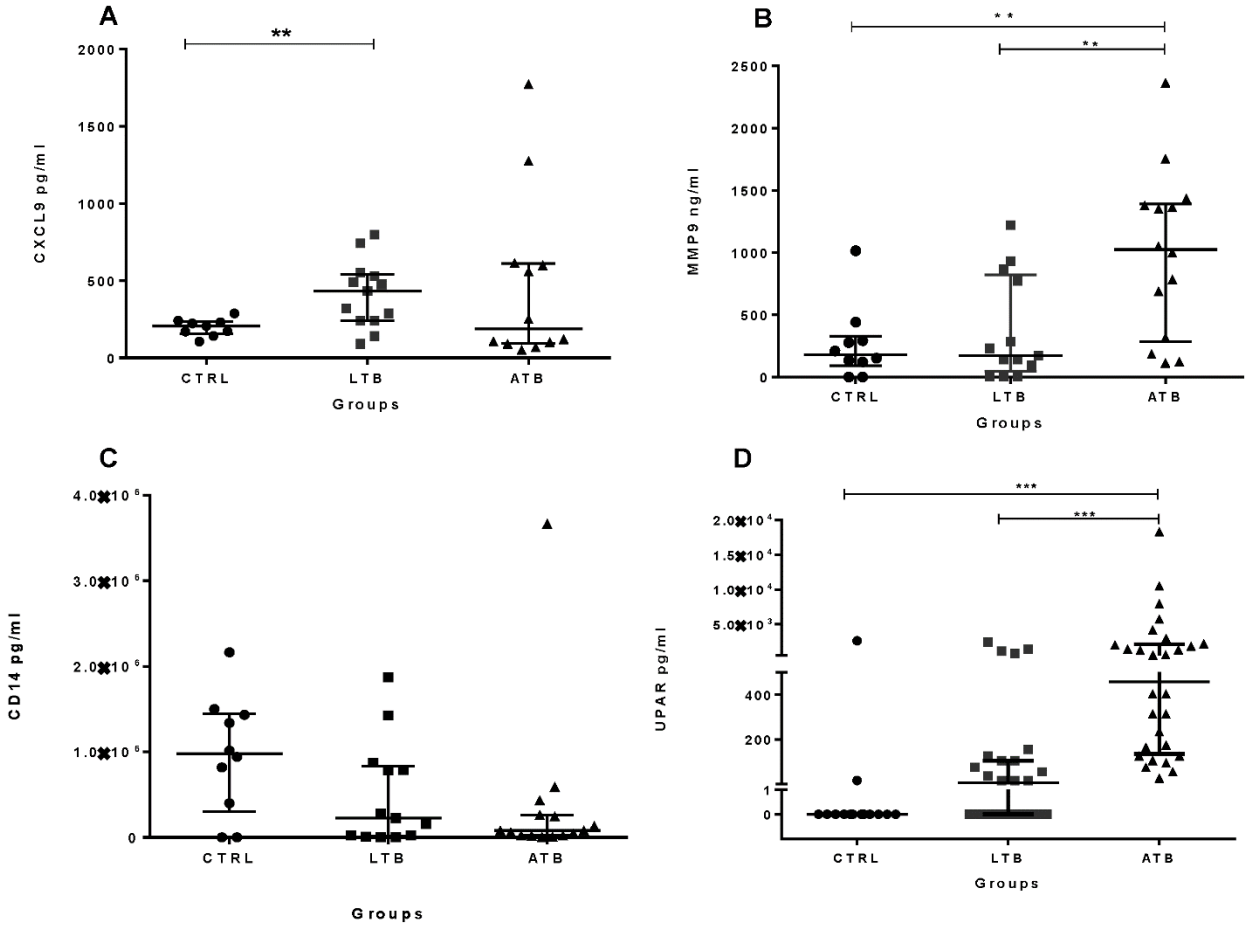


± 611,727.2 pg/mL for LTB and 960,988.3 ± 690,158.4 pg/mL for CTRL; no statistically significant differences were observed among the groups (Figure 2C). For the uPAR biomarker, serological values were 2,258.89 ± 4,052.09 pg/mL for ATB, 230.91 ± 557.80 pg/mL for LTB and 187.01 ± 694.87 pg/mL for CTRL; the ATB group had significantly higher uPAR concentrations than the LTB and CTRL groups, $p < 0.0001$ (Figure 2D).

Receiver operating characteristic (ROC) curve analysis were constructed for evaluating anti-peptide test diagnostic accuracy for discriminating patients from controls. Figure 3 shows ROC analyses. AUC ± standard error (SE) analysis were; P-12033 (AUC = 0.633 ± 0.091: 0.455-0.812 95% CI, $p < 0.162$). P-12034 (AUC = 0.728 ± 0.088: 0.555-0.901 95% CI, $p < 0.01$). P-12037 (AUC = 0.641 ± 0.090: 0.465-0.81895% CI, $p < 0.139$). P-29878 (AUC = 0.704 ± 0.083: 0.542-0.86695% CI, $p < 0.03$). Synthetic peptides P-12034 and P-29878 having cut off values of > 0.250 (OD) and > 0.150 (OD), respectively, were selected as the most appropriate for differentiating infected patient groups from non-infected subjects (Figure 3).

Biomarker concentration diagnostic power for differentiating ATB from CTRL using ROC curves comparison was also analyzed to assess diagnostic accuracy. Figure 3 shows MMP9 (AUC = 0.805 ± 0.073: 0.662-0.94995% CI, $p < 0.001$), uPAR (AUC = 0.836 ± 0.064: 0.711-0.96195% CI, $p < 0.0001$), sCD14 (AUC = 0.371 ± 0.092: 0.190-0.55195% CI, $p < 0.125$), CXCL9 (AUC = 0.412 ± 0.113: 0.190-0.63495% CI, p

Figure 2. Immunological biomarker concentrations. Optical density value distribution concerning biomarker concentrations among groups: ATB; active pulmonary TB patients (▲), LTB; subjects TST$^+$ or with latent infection (■) and CTRL; subjects TST$^-$ (●). (2A): ** represents significance of difference between the LTB and CTRL groups ($p < 0.009$) for MIG/CXCL9; (2B): ** represents significance of difference in the ATB group compared with the LTB and CTRL groups ($p < 0.005$) for MMP9. (2C): there was no significant high concentration among the groups for CD14; (2D): *** represents the significance of difference in the ATB group compared with the LTB and CTRL groups ($p < 0.0001$) for uPAR.

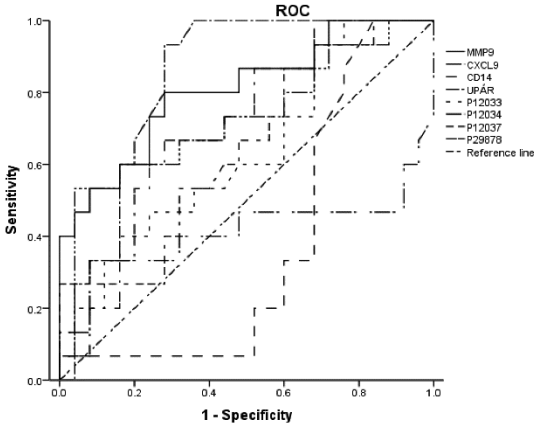


< *0.357*). MMP9 (cut off >105.5 ng/mL) and uPAR (> 31.9 pg/mL) biomarkers were selected as being the most appropriate for differentiating ATB subjects from LTB and CTRL subjects (Figure 3).

Table 2 illustrates the sensitivity and specificity of the best serological biomarker combinations. Sensitivities ranged between 100.0% (P-29878/MMP9) and 76.92% (P-12034/P-29878), while specificities ranged between 86.05% (P-12034/uPAR) and 39.13% (P-12034/MMP9). The P-29878/MMP9 biomarker combination provided the highest sensitivity (100%) for diagnosing TB, but had 52.17% specificity for detecting subjects without TB. Positive predictive value was 56.00 (34.54-77.46) and negative predictive value 100.0 (95.83-100.0). Positive likelihood ratio was 2.09 (1.36-3.20) and negative likelihood ratio0. The Youden index was 0.52 (0.32-0.73) and P-29878/MMP9 combination cut off value was 0.150/105.5 (Table 2). The P-29878/MMP9 biomarker combination diagnostic power was validated to distinguish ATB subjects from CTRL subjects using ROC curve comparison (AUC = 0.704 ± 0.083/0.805 ± 0.073: 0.542-0.866/0.662-0.94995% CI, $p < 0.03/0.001$), (Figure 4A).

Although most serological biomarker combinations were assayed, 2 tests detected the largest amount of patients, having improved sensitivity and specificity levels. The P-12034/uPAR biomarker combination provided higher sensitivity for TB diagnosis (96.67%) and 86.05% specificity, thereby detecting individuals without TB. Positive predictive value was 82.86 (68.94-96.77) and negative predictive value 97.37 (90.96-100.0). Positive likelihood ratio was 6.93 (3.29-14.16) and negative likelihood ratio 0.04. The Youden index was 0.83 (0.71-0.95) and the cut-off value 0.250/31.9 (Table 2). The P-12034/uPAR biomarker combination diagnostic power was validated to distinguish active tuberculosis from controls by analyzing ROC curves (AUC = 0.728 ± 0.88/0.836 ± 0.064: 0.555-0.901/0.711-0.96195% CI, $p < 0.01/0.0001$), (Figure 4B).

Figure 3. Receiver operating characteristics curve (ROC) analysis of host biomarker tests. ROC analysis of simple host biomarkers for discriminating TB patients from controls. The area under the curves (AUC), standard error (SE), 95% confidence interval (95% CI) of non-specific biomarkers; MMP9, uPAR, sCD14 and CXCL9 and specific biomarkers; synthetic peptides derived from the Ag85A sequence: 29879 and ESAT-6: P-12033, P-12034 and P-12037 peptides.



Marker	AUC	Standard Error	Value p	CI 95%
P-12033	0.633	0.091	0.162	0.455-0.812
P-12034	0.728	0.088	0.017	0.555-0.901
P-12037	0.641	0.090	0.139	0.465-0.818
P-29878	0.704	0.083	0.033	0.542-0.866
CD14	0.371	0.092	0.125	0.190-0.551
CXCL9	0.412	0.113	0.357	0.190-0.634
MMP9	0.805	0.073	0.001	0.662-0.9949
uPAR	0.836	0.064	0.0001	0.711-0.961

The P-12033/uPAR biomarker combination gave high sensitivity (96.67%) for TB diagnosis, 81.40% specificity, thus detecting individuals without TB. Positive predictive value was 78.38 (63.76-92.99) and negative predictive value 97.22 (90.47-100.0). Positive likelihood ratio was 5.20 (2.77-9.74) and negative likelihood ratio 0.04 (0.01-0.28). The Youden index

Table 2. Combination tests selected as appropriate for differentiating patients from controls. Sensitivity, specificity, positive and negative predictive values, likelihood ratio positive (+) and negative (-), Youden Index and cut off values.

	P-29878/MMP9	P-12034/uPAR	P-12033/uPAR
Sensitivity (%)	100.0 (96.43-100.0)	96.67 (88.58-100.0)	96.67 (88.58-100.0)
Specificity (%)	52.17 (29.59-74.76)	86.05 (74.53-97.57)	81.40 (68.60-94.19)
Positive predictive value (%)	56.00 (34.54-77.46)	82.86 (68.94-96.77)	78.38 (63.76-92.99)
Negative predictive value (%)	100.0 (95.83-100.0)	97.37 (90.96-100.0)	97.22 (90.47-100.0)
Likelihood ratio +	2.09 (1.36-3.20)	6.93 (3.29-14.6)	5.20 (2.77-9.74)
Likelihood ratio –	0.0	0.04 (0.01-0.27)	0.04 (0.01-0.28)
Youden index	0.52 (0.32-0.73)	0.83 (0.71-0.95)	0.78 (0.65-0.91)
Cut off	0.15/105.5	0.25/31.9	0.14/31.9

was 0.78 (0.65-0.91) and the cut off value 0.140/31.9 (Table 2). The P-12033/uPAR biomarker combination diagnostic power was also validated to discern between ATB and CTRL by analyzing ROC curves (AUC = 0.633 ± 0.091/0.836 ± 0.064: 0.455-0.812/0.711-0.96195% CI, $p < 0.162/0.0001$), (Figure 4C).

Biomarker accuracy for detecting subclinical TB or LTB individuals was also evaluated; Figure 5 illustrates serological biomarker combination sensitivity and specificity for separating LTB individuals from controls. The P-29878/MMP9 biomarker combination had low sensitivity (61.54%) and specificity (50.0%). Positive predictive value was 61.54 (31.25-91.83) and negative predictive value 50.0 (14.01-85.99). Positive likelihood ratio was 1.23 (0.58-2.62) and negative likelihood ratio 0.77 (0.30-1.94). The Youden index was 0.12 (0.0-0.52) and the cut off value 0.146/48.5 (Figure 5). ROC curve analysis of the P-29878/MMP9 combination was used to assess diagnostic power in differentiating LTB individuals from CTRL (AUC = 0.520 ± 0.118/0.760 ± 0.097: 0.289-0.751/0.571/0.949 95% CI, $p < 0.86/0.03$) (Figure 5A).

The P-29878/uPAR biomarker combination also had low sensitivity (46.43%) and specificity (86.67%). Positive predictive value was 86.67 (66.13-100.0) and negative predictive value 46.43 (26.17-66.69). Positive likelihood ratio was 3.48 (0.90-13.43) and negative

Figure 4. Diagnostic accuracy of the best biomarker combination tests and optical density value distribution concerning biomarker concentrations; active pulmonary TB patients (▲); LTB; subjects TST$^+$ or with latent infection (■) and CTRL; subjects TST$^-$ (●). Combination tests: (4A): P-29878/MMP9. (4B): P-12034/uPAR and (4C): P-12033/uPAR.

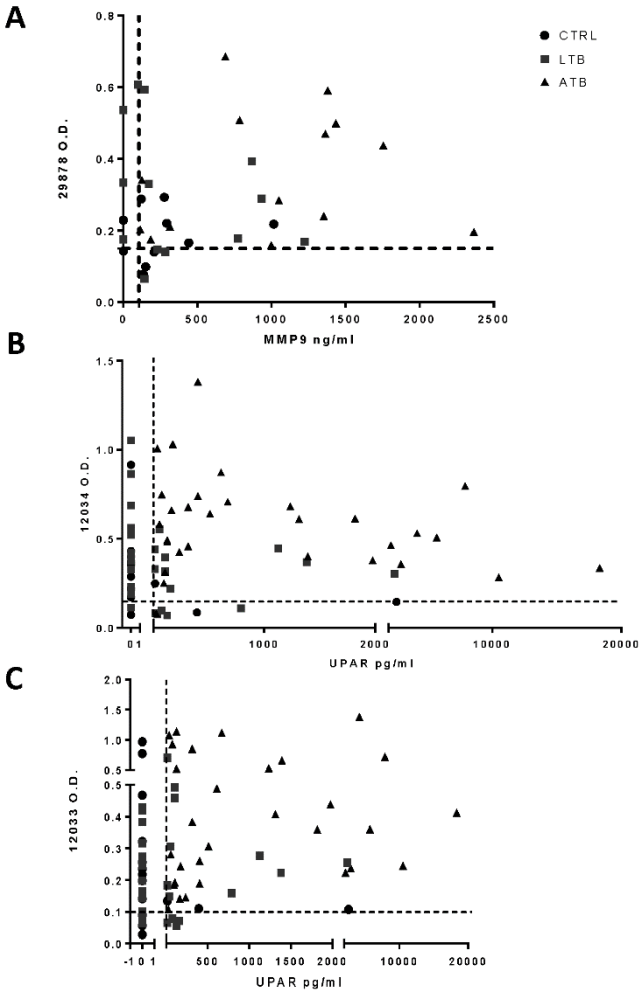
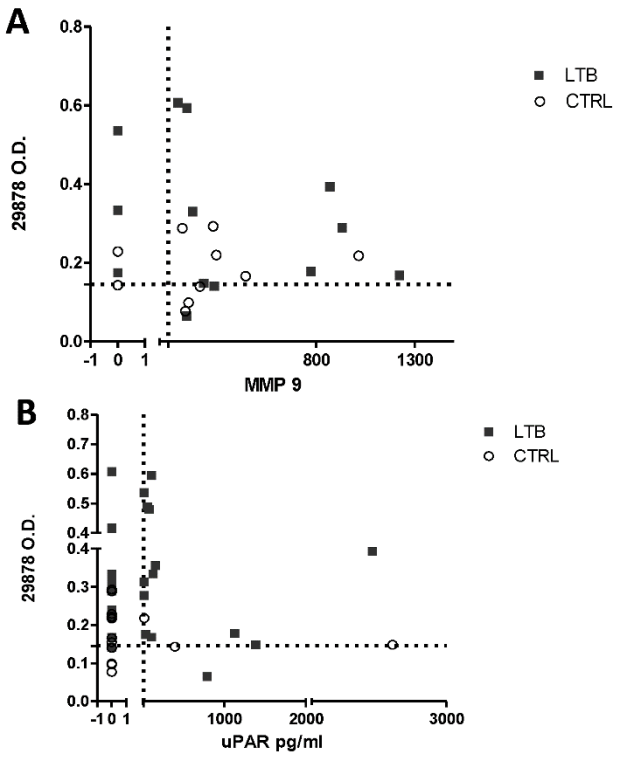


Figure5. Diagnostic accuracy of the best biomarker combination tests and optical density value distribution concerning biomarker concentrations in subclinical TB subjects. LTB; subjects TST$^+$ or with latent infection (■) and CTRL; subjects TST$^-$ (●). Combination tests: (5A): P-29878/MMP9. (5B): P-29878/uPAR.



	P-29878/MMP9	P-29878/uPAR
Sensitivity (%)	61.54 (31.25-91.83)	46.43 (26.17-66.69)
Specificity (%)	50.00 (14.01-85.99)	86.67 (66.13-100.0)
Positive Predictive Value (%)	61.55 (31.25-91.83)	86.67 (66.13-100.0)
Negative Predictive Value (%)	50.00 (14.01-85.99)	46.43 (26.17-66.69)
Likelihood Ratio +	1.23 (0.58-2.62)	3.48 (0.90-13.43)
Likelihood Ratio -	0.77 (0.30-1.94)	0.62 (0.42-0.92)
Youden Index	0.12 (0.0-0.52)	0.33 (0.08-0.58)

likelihood ratio 0.62 (0.42-0.92). The Youden index was 0.33 (0.08-0.58) and the cut off value 0.146/8.5 (Figure 5). As before, diagnostic power for discerning LTB individuals from controls was validated by ROC curve analysis (AUC = 0.661 ± 0.088/0.829 ± 0.061: 0.489-0.833/0.708-0.94995% CI, $p < 0.08/0.0001$), (Figure 5B).

Discussion

This study dealt with host serological biomarkers expressed in response to *M. tuberculosis*-active infection and their potential use for detecting active tuberculosis when combined. Biomarkers were selected based on previous transcriptomic signatures, which could be characteristic of latent or active disease. Several transcriptional immune biomarkers detected *ex vivo* have been evaluated in adult individuals having a diagnosis of TB, LTB or TST^+ and TST^- or healthy individuals, using a Custom Array 4X2K "UIMZ-IMSS-MX microarray [15]. That study has shown that serum proteins produced by some of these genes were over-expressed in whole blood from the ATB patients, making them excellent candidates for serum biomarkers since their elevation can be used to differentiate the ATB patients from the LTB or the CTRL individuals without infection [15]. Four of the 108 genes identified in the ATB subjects (the urokinase-type plasminogen activator receptor (uPAR/PLAUR), matrix metalloproteinases (MMP-9), the monokine induced by IFN-γ (MIG/CXCL9) and the soluble phosphatidylinositol-linked membrane glycoprotein (sCD14) were selected for their ability to over-express serum proteins, having high expression levels in amplification assays by RT-RCR or qPCR [15], as reported recently for MMP9 [13].

uPAR/PLAUR and MMP9 had high sensitivity and specificity values in the present work when detected in ELISA assays. The highest uPAR serological concentration was in the ATB group (2,258.89 ± 4,052.09 ng/mL), followed by the LTB group (230.91 ± 557.80 ng/mL) and the CTRL group (187.01 ± 694.87 ng/mL). The ATB group had significantly higher uPAR concentrations than the LTB and CTRL groups. The urokinase-type plasminogen activator (uPA) is secreted by polymorphonuclear neutrophils (PMN) and macrophages; uPA binds to a membrane receptor (uPAR) [23], which is expressed in cell types such as macrophages, monocytes, endothelial cells and neutrophils. uPAR can cause or modulate various diseases in cancer patients, and those suffering from various infectious and inflammatory diseases, including HIV, tuberculosis, liver fibrosis and inflammatory bowel disease [23]. uPAR can convert plasminogen to plasmin which degrades fibrin, activates matrix metalloproteases and mediates extracellular matrix protein proteolysis during cell invasion. uPAR also contributes to cell adhesion, migration, proliferation, inflammation, chemotaxis, proteolysis, immune system activation, tissue remodeling and signal transduction [23]. uPAR potential as a general biomarker has been reported in the diagnosis, prognosis and follow-up of lung disease therapy; TB patients usually have higher uPAR expression than controls [24]. The present results correlate with the forgoing as the ATB group had higher uPAR concentration (2,258.89 ± 4,052.09 ng/mL) than the CTRL group (187.01 ± 694.87 ng/mL).

Regarding MMP, high MMP9 concentration was also detected; 993.79 ± 670.87 ng/mL in the ATB group compared to 373.99 ± 419.15 ng/mL in the LTB group and 264.59 ± 296.13 ng/mL in the CTRL group. The ATB group had significantly higher MMP9 concentration than the LTB and CTRL groups. Recent studies have shown that MMPs are induced by *Mtb* during pulmonary infection [25].Underlying matrix destruction mechanisms in TB remain poorly understood but consideration of the lung extracellular matrix predicts that MMPs will play a central role owing to their unique ability to degrade fibrillar collagens and other matrix components. A critical role has been reported for MMPs in TB pathogenesis, as regulatory pathways drive MMPs, and it has been proposed that inhibiting MMP activity is a realistic goal as adjunctive therapy for limiting TB immunopathology [25]. Findings have supported the idea that early MMP activity is an essential component of resistance to pulmonary mycobacterial infection and that MMP9 is specifically required for recruiting macrophages and tissue remodeling to enable the formation of tight, well-organized granulomas [26].

MIG/CXCL9 concentration was found not to be significant among the ATB group, probably due to other biomarkers being expressed at higher levels than MIG/CXCL9. It has been reported that several other markers (i.e. IP-10, IL-2, MCP-1, MCP-2 IL-1RA and MIP-1β) are strong biomarkers, most being induced at levels many fold higher than IFN-γ [11]. MIG/CXCL9 is mainly expressed by monocytes and macrophages whereas MIG/CXCL9 is strongly induced by IFN-γ, but not IFN-α/β or other T-cell cytokines involved in IP-10 release. TNF-α is incapable of inducing MIG/CXCL9 alone, but does synergize with IFN-γ [12]. IFN-γ is crucial in leukocyte– endothelial interaction

orchestration *in vivo* and the attraction of immunocompetent cells to inflammation sites. IFN-γ synchronizes this process by up regulating adhesion molecule expression and multiple chemokine secretion, including IFN-γ-induced protein 10 (IP-10), monocyte chemotactic protein-1 (MCP-1), monokine induced by IFN-γ (MIG), macrophage inflammatory protein (MIP)-1α/β and regulated on activation, normal T-cell expressed and secreted RANTES, many seeming promising IFN-γ substitutes in immunodiagnostic tests [27,28]. MIG/CXCL9 is specifically induced by *Mtb* antigen stimulation *in vitro* and its secretion follows a similar pattern and has high correlation with IFN-γ and IP-10 [12]. MIG/CXCL9 has been shown to be expressed by IFN-γ stimulated mononuclear cells and to attract activated T-cells through chemokine receptor CXCR3; results have shown that MIG/CXCL9 production significantly correlates with enhanced T-cell IFN-γ production induced by *M. tuberculosis*-specific antigens ESAT-6/CFP-10 [27]. Even though that in the present work, MIG/CXCL9 concentration was found not to be significant within the ATB group, significantly higher MIG/CXCL9 concentration was detected in the LTB group than in the CTRL group; thus further studies should evaluate the diagnostic accuracy of this biomarker for separating subclinical TB from controls.

CD14 appears to be the most specific human monocyte marker and has been shown to be an endotoxin receptor (lipopolysaccharide) [29]. It is mainly expressed on the surface of peripheral blood monocytes, macrophages and activated granulocytes (mCD14) and its soluble form (sCD14) is present in human serum, urine and other body fluids. sCD14 biomarker concentration was assessed here; the findings showed high CD14 serological concentrations both in the CTRL group (960,988.3 ± 690,158.4 pg/mL) as well as in the LTB group (497,444.0 ± 611,727.2 pg/mL) compared to the ATB group (378,717.5 ± 925,201.2 pg/mL). No significantly different concentrations were found in the groups studied here, probably due to high standard deviations being found. The present observations must thus be validated in other studies.

sCD14 levels have been reported in bronchoalveolar lavage (BAL) fluid from pulmonary tuberculosis (PTB), an inflammatory disease characterized by granuloma formation. BAL was performed on 12 patients with active but untreated PTB and 12 healthy people. sCD14 was measured by a sandwich enzyme-linked immunosorbent assay; sCD14 level in PTB patients was increased compared to controls. No correlation was found between sCD14 levels and BAL cell differentials or lymphocyte surface markers [30].The present findings for MIG/CXCL9 and CD14 immune biomarkers did not prove to be efficacious in association with active *Mtb* infection in a sample of Venezuelan Creole population.

The World Health Organization calls for an accurate and rapid test for TB diagnosis; host biomarkers reflecting on-going pathological processes across the spectrum of *Mtb* infection and disease may have great promise [31]. Investments have yielded some progress regarding the development of new diagnostic methods, although the existing pipeline is limited for tests for sputum-smear-negative cases, childhood tuberculosis and accurate prediction of LTB reactivation. Regarding the choice of antigens central to *Mtb* immunodiagnosis, previous results using ESAT-6 (considered among the most immunogenic proteins) showed that Creole adult patients reacted significantly with residues contained in two ESAT-6 peptides [P-12033 containing residues 1 to 20 (MTEQQWNFAGIEAAASAIQG) and P-12034 containing 21 to 40 (NVTSIHSLLDEGKQSLTKLA)] [7]. The results highlighted the potential of these two ESAT-6 peptides for diagnostic applications in the Venezuelan Creole population.

Tested biomarker combinations were explored, taking into account that antigen mixtures (i.e. ESAT-6, CFP10 and TB7.7) have currently been selected for their high *Mtb* immunogenicity and specificity. ELISA screening identified combinations of 3 synthetic peptides [P-29878 (Ag85A antigen), P-12033and P-12034 (ESAT-6 antigen)] and uPAR and MMP9 proteins accurate for TB diagnosis. Biomarker combination tests were performed and analyzed by ROC curves, evaluating test sensitivity and specificity to improve early diagnosis of the ATB patients; the overall sensitivity of the 6 best immunological tests chosen for evaluating the ATB group was between 76.92% and 100.0%.

Optimal diagnostic biosignature was observed in three-biomarker combinations (P-12034/uPAR, P-12033/uPAR and P-29878/MMP9). P-12034/uPAR having 96.67% sensitivity and 86.05% specificity, P-12033/uPAR having 96.67% sensitivity and 81.40% specificity and P-29878/MMP9 with 100% sensitivity and 52.17% specificity. P-12034/uPAR was especially able to differentiate study groups better, demonstrating that these molecules are good candidates as serological biomarkers for diagnosing ATB and have proved to be efficacious in association with ATB infection.

Before addressing the question of whether potential novel immunodiagnostic markers can improve the management of individuals having presumed LTB, we explored whether the biomarkers studied in the present work correlated with incipient TB as their prevalence should be higher in asymptomatic TST^+ subjects who could be at a high-risk for TB. The diagnostic power of immune biomarkers to distinguish subclinical TB from the ATB and CTRL individuals was evaluated by comparing ROC curves. The present study demonstrated the presence of these biomarkers during incipient subclinical TB; however, they had low sensitivity (61.54% and 39.29% for P-29878/MMP9 and P-12034/uPAR, respectively). Immune biomarker combinations did not prove to be efficacious in association with LTB.

Many attempts have been made to develop a serological TB test that can discriminate ATB from LTB, avoid cross-reactivity to Bacille Calmette-Guérin (BCG) or non-tuberculous mycobacteria and perform consistently in genetically and immunologically diverse populations [32]. The complexity of *Mtb* biology and interactions with a human host have impeded the development of biomarkers that can distinguish between ATB and LTB or other diseases, especially in HIV-infected adults and children [32,33]. Actual LTB tests do not adequately distinguish resolved from persistent infection and are unable to efficiently identify individuals who are at the highest risk of reactivation [10,11]. Studies of interferon (IFN)-γ release assay (IGRAs) predictive values have only shown modest predictive ability and several studies have shown similar (and rather low) progression rates in people having positive TST and IGRA test results.

IGRAs were designed to address low TST specificity, thus providing more accurate diagnosis and better prediction of progression to active TB. Several new markers have been suggested as specific for tuberculosis or LTB and indicating a high risk of progression to active tuberculosis; however, relevant data are preliminary. A 2-year follow-up study of *Mtb* antigen-driven IFN-γ responses and sCD14 receptor serum levels has been reported recently, involving 60 healthy Polish adults having recent household or long-term work TB contact and individuals without known *Mtb* exposure [29]. All of them underwent baseline and repeated testing with IGRA and serum sCD14 ELISA quantification. IGRA reversions were noticed in almost one-third of work TB contacts but not in participants from the household TB contact group. IGRA conversions were found in 40% of household TB contacts. No correlation was found between the IGRA results and the sCD14 levels. Monitoring serum sCD14 level can reduce the likelihood of a false prediction of active TB development in close TB contacts showing an *M. tuberculosis*-specific increase in IFN-γproduction in repeated IGRA testing [29]. A recent report on identifying potential immune biomarkers able to distinguish active TB from LTB has suggested that IL-17F, MIP-3α, IL-13, IL-17A, IL-5, IL-9, IL-1β, IL-2 and IFN-γ could identify and uniquely discriminate between TB states [34]. It has been reported that the use of ESAT-6- and CFP-10-based IGRAs may underestimate LTB incidence whereas the use of heparin-binding hemagglutinin adhesin (HBHA) may combine the operational advantages of IGRAs with high sensitivity (92.06 %) and specificity (93.88 %) for LTB [35].

Our study identified several interesting potential host biomarkers for detecting *Mtb*-non-specific and specific immune responses (uPAR and MMP9 and anti-P-12034, -P-12033 and –P-29878). Three biomarker combinations validated for their diagnostic power indiscriminating ATB subjects from non-ATB cases (P-12034/uPAR, P-12033/uPAR and P-29878/MMP9 combination tests), had high sensitivity and specificity. The P-12034/Upar combination was the most promising alternative immunodiagnostic biomarker, showing higher accuracy for diagnosing infection in HIV^-TB^+ individuals. We have thus identified a biosignature for 5 biomarker host serum proteins for TB diagnosis. The P-12034/uPAR combination has potential for further development and validation and further studies should evaluate the diagnostic accuracy of the proposed biomarkers and their usefulness in monitoring the response to TB treatment.

Conclusions

The current work has investigated the CXCL9, CD14, MMP9, uPAR and anti-ESAT-6 and –Ag85A synthetic peptide accuracy as host serological biomarkers; the findings demonstrated that the anti-P-12034/uPAR combination could have potential for identifying clinical TB patients. This approach holds promise for further validation with a view to progress in developing new tools for rapid TB diagnosis, especially in sputum-smear-negative cases and childhood tuberculosis in resource-constrained settings, as in our countries (Venezuela-Colombia-Mexico).

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