IMMUNO-DIAGNOSIS OF ACTIVE TUBERCULOSIS

EDITED BY: Harriet Mayanja-Kizza, Pere-Joan Cardona and Novel N. Chegou

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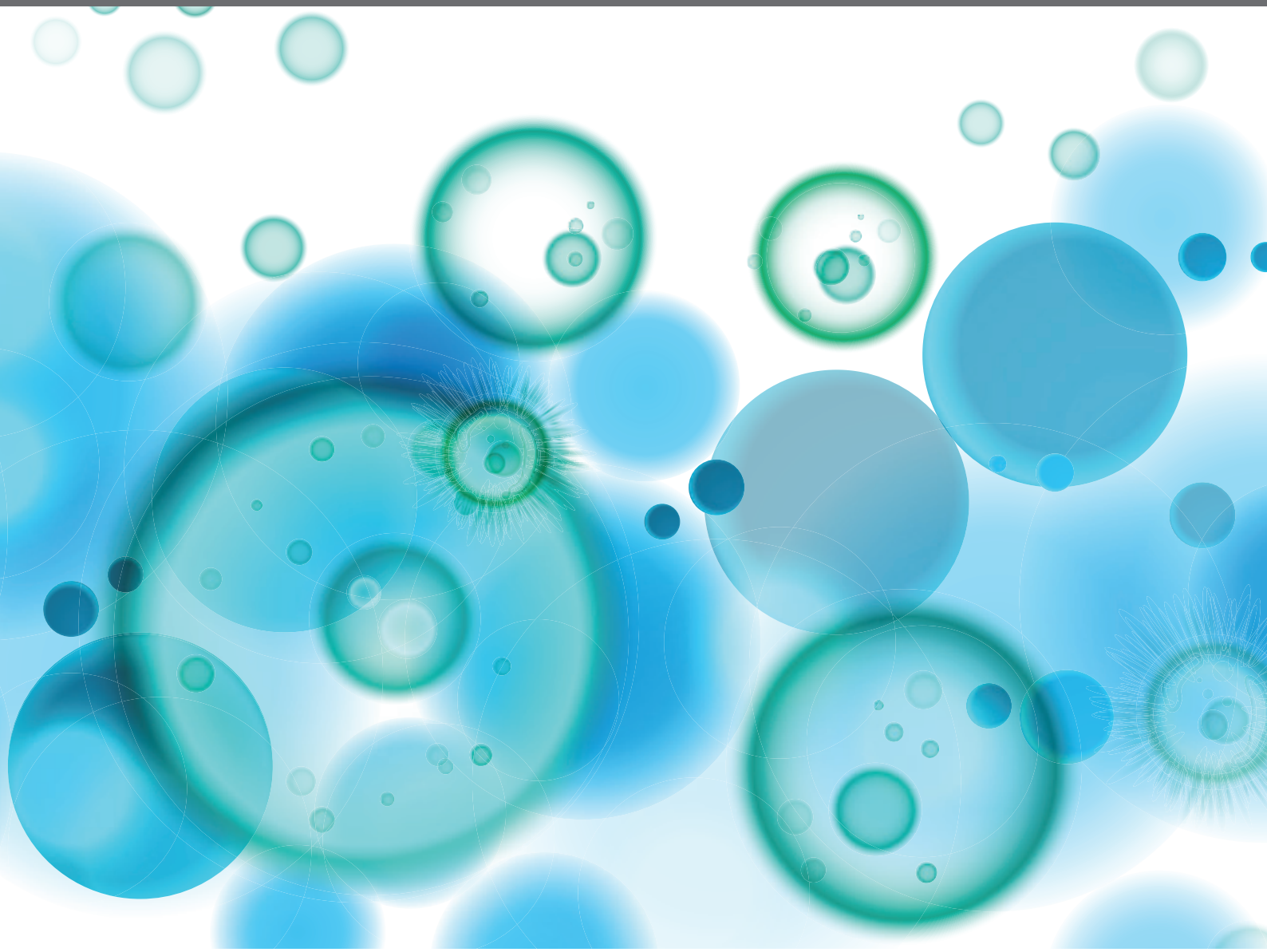

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IMMUNO-DIAGNOSIS OF ACTIVE TUBERCULOSIS

Topic Editors:
Harriet Mayanja-Kizza, Makerere University, Uganda
Pere-Joan Cardona, Hospital Universitari Germans Trias i Pujol, Spain
Novel N. Chegou, Stellenbosch University, South Africa

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Table of Contents

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Ian Marriott,
University of North Carolina at Charlotte,
United States

*CORRESPONDENCE
Harriet Mayanja-Kizza
hmk@chs.mak.ac.ug

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Editorial: Immuno-diagnosis of active tuberculosis; are we making progress?

Harriet Mayanja-Kizza[1,2]*, Pere-Joan Cardona[3,4,5,6] and Novel N. Chegou[7,8]

[1]Makerere University, Kampala, Uganda, [2]Uganda CWRU Research Collaboration, Kampala, Uganda, [3]Department of Microbiology, University of Barcelona, Barcelona, Spain, [4]Microbiology Department, North Metropolitan Clinical Laboratory, 'Germans Trias i Pujol' University Hospital, Badalona, Catalonia, Spain, [5]Genetics and Microbiology Department, Universitat Autònoma de Barcelona, Cerdanyola del Vallès, Catalonia, Spain, [6]Centro de Investigación Biomédica en Red de Enfermedades Respiratorias (CIBERES), Madrid, Spain, [7]DSI-NRF Centre of Excellence for Biomedical Tuberculosis Research South African Medical Research Council Centre for Tuberculosis Research, South Afriacan, South Africa, [8]Division of Molecular Biology and Human Genetics, Department of Biomedical Sciences, Faculty of Medicine and Health Sciences, Stellenbosch University, Cape Town, South Africa

KEYWORDS

tuberculosis, immuno-diagnosis, current trends, potential markers, future directions

Editorial on the Research Topic
Immuno-diagnosis of active tuberculosis

Active tuberculosis (ATB) diagnosis suffers from several knowledge gaps and challenges, including the need for newer assays with faster turn-around-time (TAT), high sensitivity and specificity, lower costs, and the potential for Point-of-Care (POC) use assays. Towards this goal, we need novel tests that can be used at the population level to screen and/or test high risk populations, including household contacts of sputum positive index patients and persons living with HIV/AIDS. In addition, tests that can be adaptable towards use in treatment follow-up (to predict cure or relapse/reinfection), and as potential surrogate markers of tuberculosis (TB) vaccination are important towards the global control of tuberculosis. A test using blood-based samples would have potential for future studies to "estimate" the mycobacterial load in the body (akin to the HIV viral load test), which would improve the treatment of ATB, and latent tuberculosis infection (LTBI). In addition, immune-based POC tests for ATB diagnosis have the potential for low invasiveness, being user friendly for staff and patients, minimal training needs, greater simplicity, and low cost; making immuno-diagnosis of active tuberculosis a promising approach. To date, a number of studies have been done in both high and low TB endemic settings to diagnose, or even predict, the evolution towards ATB. Much as some studies have shown high sensitivity and specificity (over 85%), there are still many challenges towards the ideal test, and more research needs to be done.

Studies for an Immune marker in active tuberculosis diagnostics

Immunodiagnostic studies to diagnose and/or predict the possibility of pulmonary tuberculosis (PTB have recently been conducted in different non sputum specimens, including serum, plasma, unstimulated or stimulated Quantiferon supernatants, peripheral blood mononuclear cells (PBMC), in addition to extra-sanguineous specimens, such as saliva, urine, pleural fluid, ascites, cerebrospinal fluid as well as bronchio-alveolar lavage cells and fluids, and induced sputum. The current literature still shows limited sensitivity and specificity in the diagnosis of ATB, with studies showing variable markers predicting ATB, despite generally related assays and study populations; with few achieving optimal sensitivity and specificity.

Namuganga and colleagues compared immune markers in saliva and sputum among patients with Genexpert confirmed ATB, and showed different significant markers between saliva and serum, thus highlighting the potential for saliva-based ATB diagnostic markers (1). Two multi-country studies in high ATB endemic African sites showed potential of Mtb host response markers in different combinations with varying levels of sensitivity and specificity (up to 94 and 73% respectively) either in predicting ATB compared to healthy controls, (or better still) to Non-MTB respiratory infections (2, 3). However, these studies required 7 and 4 host marker combinations, respectively, to attain the high sensitivity observed, making them expensive to produce for population based ATB triage assays. Moreover, there was little overlap of significant host markers (only CRP and IP-10) in these 2 studies, indicating possible site, technique or individual variation, impacts on the results obtained for particular markers.

In this collection, Luo et al. show that a single candidate marker HLA-DR on Mtb responding specific cells could differentiate ATB from LTBI with a high area under the ROC (receiver operating characteristic) curve, (0.901), and high sensitivity, but moderate specificity, a step towards a low cost potential triage test marker. Furthermore, the same group shows that HLA-DR on Mtb-specific cells, combined with TBAg/PHA ratio increased the ROC to 0.937. However, not all markers are upregulated in ATB, as in the paper by Garlant et al., where in a 6-molecule biosignature, transmembrane protein 49 (TMEM9) was downregulated in ATB compared to LTBI negative controls. In a meta-analysis, Meca et al. suggest that CRP alone, above 8 mg/L has potential as a single molecule triage diagnostic test. Related to this, high sensitivity (hs) CRP alone may differentiate various forms of pediatric ATB compared to healthy children, making this a potentially lower cost, variable age, triage test (4). Another pediatric test by Tornheim et al. suggests plasma kynurenine levels could be useful as an ATB diagnostic marker in children, having high sensitivity (81.5), but low ROC (0.667). It, would, however, be of interest to determine the performance of the combined assessment ofhsCRP and kynurenine levels in pediatric populations. Mann et al. show 4-5 high sensitivity biomarker signatures in patients with confirmed spine ATB relative to control subjects, which may diagnose other forms of extra-pulmonary tuberculosis.

In Brazil, a study by Queiroz et al. of persons with advanced HIV disease indicates the diagnostic potential of an assay with two cytokines, IL-15 and IL-10, in plasmathat are lower in ATB compared to those that received early anti ATB treatment. This emphasizes the possible role of low-level biomarkers in certain disease states, a fact that is often overlooked, or ignored as a "negative result" as increased levels in a biosignature are often expected in diagnostic studies.

Among pregnant women with active tuberculosis, Ranaivomanana et al.'s find lower IGRA responses, probably related to the protective reduction in inflammatory immunity during pregnancy, a finding of relevance to studies in pregnant women.

Serology tests

Serology has over the years been considered an attractive low cost option for the detection of ATB, with a number of products on the market, and used in low income high endemicity countries, despite most not being validated, or recommended for use in the general population. However, it is thought that some serological tests can be used as adjunctive diagnostic tests in HIV-infected patients with smear-negative PTB or extra-PTB, despite their current limitations (5). Although serum antibodies to ESAT-6 and CFP-10 were considered an attractive option to differentiate between LTBI and ATB, a related study performed in high and low ATB endemic countries, failed to show an ability to differentiate Mtb infection from PTB (6). Another sero-diagnostic study combining four MTB antigens ESAT-6, CFP-10, CFP-21, and MPT-64 did not significantly differentiate PTB from LTBI (7). However, an antibody study in South Africa shows promise when used in combination with cytokine markers (8). Recently, a new promising serological lateral flow assay (LFA) has entered the market (LIODetect®TB-ST). This POC test detects IgG, IgA and IgM antibodies against purified recombinant protein antigens PstS1 and PstS3 together with highly purified lipoglycan of Mtb cell wall in serum, plasma or whole blood within 20 min (9). Intrestingly, Nziza et al. show a serological multiplex assay including 7 MTB antigens and 3 novel antibodies that is able to discern LTBI and ATB across HIV positive and negative populations.

Other potential markers

In a 16-article meta-analysis, the ovarian cancer tumor marker CA-125 has shown promise for detection of ATB with a pooled sensitivity of 0.85 (10). This highlights the importance of thinking outside the box, to identify novel biomarkers and the potential of usingbiomarkers associated with other disease conditions to diagnose ATB.

Future directions

Overall, there has been extensive research towards an ideal test for immunodiagnosis of ATB. However, there is a need for more research with innovative approaches. These could include further studies looking at cell-mediated responses, with various cytokine/chemokine responses, in different combinations, and the possibility of combining these with antibody responses. In addition, costs should be considered by developing tests with the lowest possible number of biomarkers, as well as considering the availability for POC assays at a population level.

Other options could include blood transcriptomic signatures and blood immune-profiling, immune metabolic markers (e.g. endocrine markers, and pro-inflammatory markers), the use of novel antigens, or combinations of different antigen stimulation responses. Also, studies using body samples other than blood, like saliva, sputum, exudative fluids, bronco alveolar lavage (BAL), breath condensate, urine, and pleural, ascitic or pericardial fluids, could lead to novel immune markers for ATB diagnosis(pulmonary and extra pulmonary). In addition, different diagnostic approaches, such as LFA-POC tests, and other techniques that are faster and have lower costs, diagnosis lower cost test need to be further investigated.

Author contributions

All authors listed have made a substantial, direct, and intellectual contribution to the work and approved it for publication.

Conflict of interest

The authors declare that the research was conducted in the absence of any commercial or financial relationships that could be construed as a potential conflict of interest.

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ORIGINAL RESEARCH
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Activation Phenotype of *Mycobacterium tuberculosis*-Specific CD4$^+$ T Cells Promoting the Discrimination Between Active Tuberculosis and Latent Tuberculosis Infection

Ying Luo[1], Ying Xue[2], Liyan Mao[1], Qun Lin[1], Guoxing Tang[1], Huijuan Song[1], Wei Liu[1], Shutao Tong[1]*, Hongyan Hou[1]*, Min Huang[1]*, Renren Ouyang[1]*, Feng Wang[1]* and Ziyong Sun[1]**

[1] Department of Laboratory Medicine, Tongji Hospital, Tongji Medical College, Huazhong University of Science and Technology, Wuhan, China, [2] Department of Immunology, School of Basic Medicine, Tongji Medical College, Huazhong University of Science and Technology, Wuhan, China

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Edited by:
Harriet Mayanja Kizza,
Makerere University, Uganda

Reviewed by:
Marco Pio La Manna,
University of Palermo, Italy
Elisa Nemes,
University of Cape Town, South Africa

***Correspondence:**
Shutao Tong
1170879123@qq.com
Hongyan Hou
815739195@qq.com
Min Huang
huangminry2013@163.com
Renren Ouyang
872442321@qq.com
Feng Wang
fengwang@tjh.tjmu.edu.cn
Ziyong Sun
zysun@tjh.tjmu.edu.cn
Ying Luo
13349917282@163.com

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Background: Rapid and effective discrimination between active tuberculosis (ATB) and latent tuberculosis infection (LTBI) remains a challenge. There is an urgent need for developing practical and affordable approaches targeting this issue.

Methods: Participants with ATB and LTBI were recruited at Tongji Hospital (Qiaokou cohort) and Sino-French New City Hospital (Caidian cohort) based on positive T-SPOT results from June 2020 to January 2021. The expression of activation markers including HLA-DR, CD38, CD69, and CD25 was examined on *Mycobacterium tuberculosis* (MTB)-specific CD4$^+$ T cells defined by IFN-γ, TNF-α, and IL-2 expression upon MTB antigen stimulation.

Results: A total of 90 (40 ATB and 50 LTBI) and another 64 (29 ATB and 35 LTBI) subjects were recruited from the Qiaokou cohort and Caidian cohort, respectively. The expression patterns of Th1 cytokines including IFN-γ, TNF-α, and IL-2 upon MTB antigen stimulation could not differentiate ATB patients from LTBI individuals well. However, both HLA-DR and CD38 on MTB-specific cells showed discriminatory value in distinguishing between ATB patients and LTBI individuals. As for developing a single candidate biomarker, HLA-DR had the advantage over CD38. Moreover, HLA-DR on TNF-α$^+$ or IL-2$^+$ cells had superiority over that on IFN-γ$^+$ cells in differentiating ATB patients from LTBI individuals. Besides, HLA-DR on MTB-specific cells defined by multiple cytokine co-expression had a higher ability to discriminate patients with ATB from LTBI individuals than that of MTB-specific cells defined by one kind of cytokine expression. Specially, HLA-DR on TNF-α$^+$IL-2$^+$ cells produced an AUC of 0.901 (95% CI, 0.833–0.969), with a sensitivity of 93.75% (95% CI, 79.85–98.27%) and specificity of 72.97% (95% CI, 57.02–84.60%) as a threshold of 44% was used. Furthermore, the performance of HLA-DR on TNF-α$^+$

IL-2$^+$ cells for differential diagnosis was obtained with validation cohort data: 90.91% (95% CI, 72.19–97.47%) sensitivity and 68.97% (95% CI, 50.77–82.73%) specificity.

Conclusions: We demonstrated that HLA-DR on MTB-specific cells was a potentially useful biomarker for accurate discrimination between ATB and LTBI.

Keywords: activation phenotype, HLA-DR, *Mycobacterium tuberculosis*, discrimination, active tuberculosis, latent tuberculosis infection

INTRODUCTION

Tuberculosis (TB), caused by *Mycobacterium tuberculosis* (MTB) infection, remains a lethal infectious disease that needs to be paid more attention globally (1–3). It was reported that there were still an estimated 10 million cases and nearly 1.4 million deaths of the disease in 2019 (4). The one main hurdle in controlling TB is the difficulty in differentiating active TB (ATB) from latent TB infection (LTBI) (5–8). Delayed ATB identification would enhance TB transmission and hamper the global End TB strategy (9, 10). Therefore, the development of tools for discrimination between ATB and LTBI would be a priority in the global effort to combat this disease (11, 12).

Pathogen detections including smear microscopy and mycobacterial culture were applied widely in the TB diagnosis field. However, the drawbacks of these conventional tests included limited sensitivity and length of time consumed (13, 14). Although a rapid diagnosis has been enhanced by various molecular techniques such as GeneXpert MTB/RIF and GeneXpert MTB/RIF Ultra (15–20), these tests also showed unsatisfactory utility, especially for paucibacillary cases (21–24). Interferon gamma release assays, including T-SPOT.TB (T-SPOT) and QuantiFERON-TB Gold In-Tube (QFT-GIT), were widely used for identifying MTB infection (25–28). Nevertheless, these two tests were not useful in distinguishing ATB from LTBI (29–31). Moreover, a variety of host biomarkers identified by omics including transcriptome (32–34), proteome (35, 36), and metabolome (37–39) have been proposed as potential biomarkers for diagnosing MTB infection. Howbeit, few studies validated their actual benefit in various areas with different demographic, ethnic settings, as well as TB prevalence. Besides, high operating costs and infrastructural requirements hinder their use in resource-constrained settings. Hence, there is an imperative need to seek an approach with good performance based on existing technology platforms.

Previous studies showed that MTB-specific T-cell activation might associate with MTB antigen load (40, 41). Several publications had denoted the potential of antigen-specific HLA-DR and CD38 in TB diagnosis (41, 42). Notwithstanding, further validation for these investigations, especially in different populations, is needed to get the most promising biomarkers a step closer to clinical translation. Meanwhile, T-cell activation could reflect by multiple markers including HLA-DR, CD38, CD69, and CD25. HLA-DR is usually highly expressed on the surface of T cells in advanced stages of activation as one kind of human class II major histocompatibility complex antigen (43–45). CD38 is a type II transmembrane glycoprotein that is extensively expressed on cells of hematopoietic and non-hematopoietic lineage (46). It is downregulated in resting memory cells and elevates in activated cells (45, 47). CD69 acts as a costimulatory molecule for T-cell activation and proliferation (48). It is one of the early markers upregulated after T-cell activation (45, 49–51). CD25, as α chain of IL-2 receptor, plays a key role in responsiveness to IL-2, enabling T lymphocyte activation and further IL-2 production (45, 52). Different activation biomarkers may have inconsistent performance in the diagnosis of TB. Besides, CD27 acts as a differentiation marker expressed on T cells as a member of the TNF receptor family (53). It was reported that the decreased expression of this marker denoted a conversion of T cells towards effector memory phenotype (54). Several previous studies have found that CD27 expression on MTB-specific cells decreased in ATB patients (40, 55, 56), suggesting its potential in TB diagnostics. Moreover, the value of the combination of various activation and differentiation biomarkers for TB diagnostics has not been adequately elaborated. Therefore, the issue is needed to be clarified. Meanwhile, MTB-specific T cells could represent by multiple cytokine secretion profiles including IFN-γ, TNF-α, and IL-2 upon MTB antigen stimulation. Thus, there is urgent need to identify appropriate combination for TB diagnosis with optimal performance. The present study evaluated and validated the potential utility of various activation and differentiation markers on different MTB-specific cells for TB diagnosis.

METHODS

Study Design

The present study was carried out between June 2020 and January 2021. Participants were recruited at Tongji Hospital (Qiaokou cohort, the largest tertiary hospital in central China with 5500 beds) and Sino-French New City Hospital (Caidian cohort, a branch hospital of Tongji Hospital with 1600 beds), respectively. Subjects in two cohorts were selected based on positive T-SPOT results. On the basis of clinical and laboratory assessments, participants were classified as patients with ATB and individuals with LTBI. ATB patients were diagnosed by positive mycobacterial culture and/or GeneXpert MTB/RIF with supportive symptoms and radiological findings of ATB. LTBI individuals were identified by positive T-SPOT result in the absence of ATB evidence. Exclusion criteria for the study included (1) having received anti-TB therapy for more than 2 weeks and (2) being younger than 17 years old. This study was reviewed and approved by the committee of Tongji Hospital,

Tongji Medical College, Huazhong University of Science and Technology.

T-SPOT Assay

Heparin-anticoagulated blood samples were collected for performing T-SPOT assay (Oxford Immunotec, Oxford, UK). The operation was conducted in accordance with manufacturer's instruction. Briefly, peripheral blood mononuclear cells (PBMCs) were separated by Ficoll-Hypaque gradient centrifugation. Then, the isolated PBMCs (2.5×10^5) were added to 96-well plates precoated with antibody against IFN-γ. There were four wells each participant: medium (negative control), early secreted antigenic target 6 (ESAT-6), culture filtrate protein 10 (CFP-10), and phytohemagglutinin (positive control). Cells were incubated for 16–20 h at 37°C with 5% CO_2 and developed using anti-IFN-γ antibody conjugate with substrate to detect the presence of IFN-γ secreted cells. Spot-forming cells were counted with an automated enzyme-linked immunospot reader (CTL Analyzers, Cleveland, OH, USA). The test result was regarded positive if ESAT-6 and/or CFP-10 spot number minus negative control spot number ≥ 6. The test result was regarded negative if both ESAT-6 spot number minus negative control spot number and CFP-10 spot number minus negative control spot number ≤ 5. Results were considered undetermined if the spot number in phytohemagglutinin well was <20 or spot number in the medium well was >10.

Detection of Markers on MTB-Specific $CD4^+$ Cells

PBMCs were stimulated with peptide ESAT-6 (2 μg/ml) and CFP-10 (2 μg/ml) for 18 h at 37°C with 5% CO_2. Briefly, PBMCs were counted and 1×10^6 cells were added to the well. Brefeldin A was added to the mixture 6 h before staining of the cells. Post incubation, PBMCs were first stained with Fixable Viability Stain 700 (BD Pharmingen) to discriminate live from dead cells, followed by appropriate surface marker staining. Cell surface staining was performed on PBMCs using the following anti-human monoclonal antibodies: anti-CD4-APC-Cy7, anti-HLA-DR-PerCp 5.5, anti-CD38-BV510, anti-CD69-BV421, anti-CD25-APC, and anti-CD27-PE-Cy7. For intracellular staining, the cells were fixed and permeabilized with Fixation and Permeabilization Buffer (BD Biosciences). Intracellular cytokine staining was conducted using the following anti-human monoclonal antibodies: anti-IFN-γ-BV605, anti-TNF-α-FITC, and anti-IL-2-PE. The staining was performed at 4°C, and the cells were stained for 30 min. The gating strategy was shown in **Figure 1**. The information of used antibodies was presented in **Supplementary Table S1**. Isotype controls with

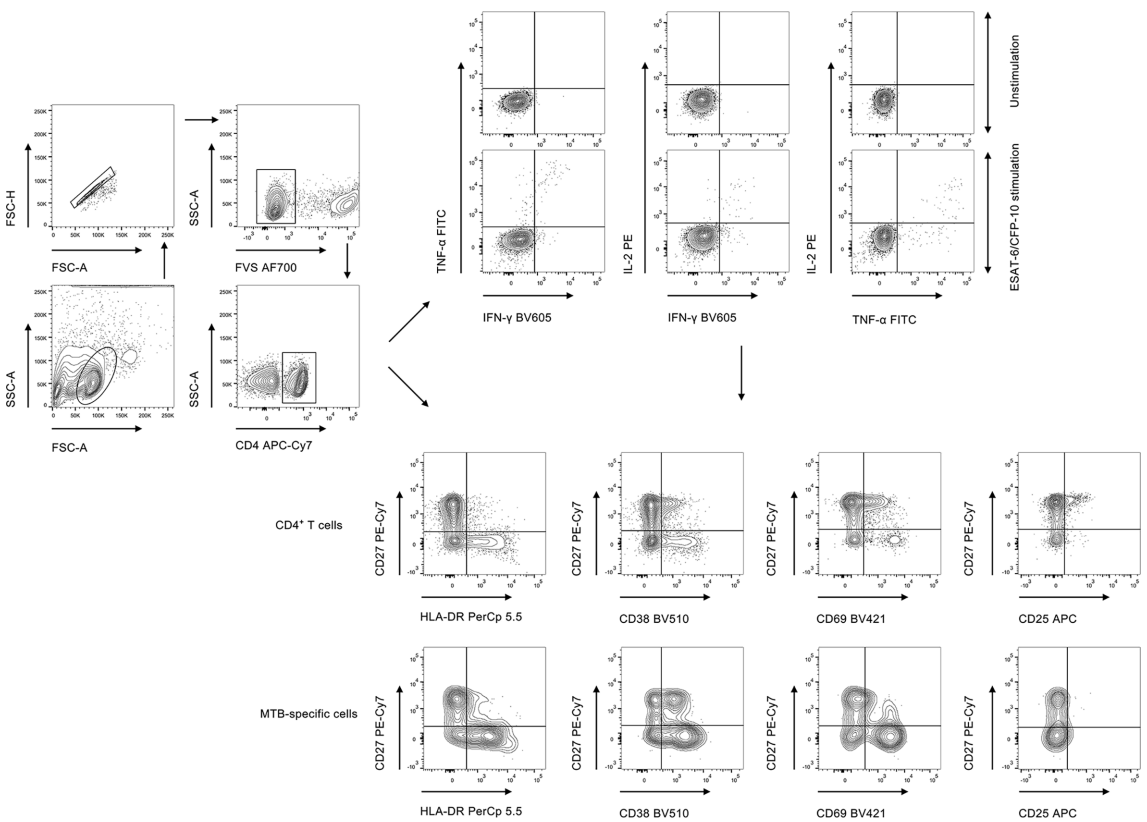

FIGURE 1 | The gating strategies for cytokine expression and activation phenotype in the current study. FVS, fixable viability stain; MTB, *Mycobacterium tuberculosis*.

irrelevant specificities were included as negative controls. After washing, the pellets were resuspended in 200 μl staining buffer and analyzed with FACSCanto II flow cytometer (BD Biosciences, San Jose, CA). The flow data were analyzed using Flowjo software version 10.6.2 (TreeStar, Ashland, OR).

The background in the unstimulated tube was subtracted when reporting the percentage of cytokine-producing cells in the stimulated tube. MTB-specific cells were determined by cytokine production including IFN-γ, TNF-α, and IL-2. Responders were defined as individuals with relative counts of cytokine-producing $CD4^+$ T cells (more than 15 events were recorded) in MTB antigen-stimulated condition that were significantly higher than the unstimulated control (a fold change of more than 3). The 15-event cut-off for phenotypic analysis was applied to each cytokine combination. Phenotype analysis for MTB-specific $CD4^+$ T cells was only performed on responders.

Statistical Analysis

Continuous variables were described using median (interquartile range) or means ± standards deviation (SD). Categorical variables were expressed as number (%). Comparison between various groups was performed using Mann-Whitney *U* test for continuous variables, and Chi-square test or Fisher's exact test for categorical variables. A two-side test with $P < 0.05$ was considered statistically significant.

The diagnostic performance of various biomarkers was assessed by receiver operator characteristics (ROC) curve analysis. Area under the curve (AUC), sensitivity, specificity, positive predictive value (PPV), negative predictive value (NPV), positive likelihood ratio (PLR), negative likelihood ratio (NLR), and accuracy, together with their 95% confidence intervals (CI), were determined. The comparison between various ROC AUCs was performed by using z test with the procedure of Delong et al. (57). Data analysis was performed using GraphPad Prism version 8 (GraphPad Software, San Diego, CA, USA), R 4.0.2 program (R Core Team), SPSS version 25.0 (SPSS, Inc., Chicago, IL, USA), and MedCalc version 11.6 (MedCalc, Mariakerke, Belgium).

RESULTS

Demographic and Clinical Characteristics of Study Participants

Out of 90 subjects from Qiaokou cohort, 40 were ATB patients and the other 50 were LTBI individuals. Among 64 cases from the Caidian cohort, 29 were diagnosed as ATB and 35 were diagnosed as LTBI. Participants had a mean age of around 60 years and more than half were males. There was no significant difference between ATB patients and LTBI individuals in distribution of age and gender. An overview of the clinical and demographic characteristics of recruited participants was presented in **Table 1**.

Cytokine Expression Patterns Upon MTB Antigen Stimulation for Distinguishing ATB From LTBI

We measured the expression of Th1 cytokines including IFN-γ, TNF-α, and IL-2 secreted by $CD4^+$ T cells upon MTB antigen stimulation and analyzed the value of expression profile for distinguishing ATB patients from LTBI individuals. It was observed that statistical difference existed in some co-expression patterns between patients with ATB and LTBI individuals (**Figure 2A**). When we assessed the ability of cytokine secretion patterns to diagnose ATB disease using ROC curve analysis, it was found that the AUCs produced by all candidates were lower than 0.7 (**Figure 2B**).

Activation Markers on $CD4^+$ T Cells for Discriminating ATB From LTBI

The utility of activation markers including HLA-DR, CD38, CD69, and CD25 on $CD4^+$ T cells for discriminating ATB patients from LTBI individuals was evaluated. The expression of CD38 and CD25 in ATB patients was significantly higher than that in LTBI individuals, while no statistical difference was observed in HLA-DR and CD69 expression between these two groups (**Figure 3A**). ROC curve analysis showed that the AUCs

TABLE 1 | Demographic and clinical characteristics of study population.

Variables	Qiaokou cohort (training set)		*P**	Caidian cohort (validation set)		*P**	*P*†
	ATB (n = 40)	LTBI (n = 50)		ATB (n = 29)	LTBI (n = 35)		
Age, years	50.8 ± 18.2	48.9 ± 16.7	0.529	52.0 ± 17.0	53.1 ± 15.7	0.981	0.351
Sex, male, %	25 (62.5%)	31 (62.0%)	0.961	17 (58.6%)	21 (60.0%)	0.911	0.721
Underlying condition or illness							
HIV infection	1 (2.5%)	0 (0.0%)	0.261	1 (3.5%)	0 (0.0%)	0.268	0.807
Diabetes mellitus	7 (17.5%)	7 (14.0%)	0.649	5 (17.2%)	3 (8.6%)	0.296	0.593
Solid tumor	5 (12.5%)	4 (8.0%)	0.48	3 (10.3%)	4 (11.4%)	0.89	0.851
Hematological malignancy	3 (7.5%)	1 (2.0%)	0.208	2 (6.9%)	1 (2.9%)	0.447	0.943
Liver cirrhosis	2 (5.0%)	1 (2.0%)	0.431	2 (6.9%)	3 (8.6%)	0.804	0.217
End-stage renal disease	5 (12.5%)	3 (6.0%)	0.282	5 (17.2%)	3 (8.6%)	0.296	0.469
Organ transplantation	2 (5.0%)	0 (0.0%)	0.11	1 (3.5%)	1 (2.9%)	0.892	0.729
Immunosuppressive condition‡	7 (17.5%)	7 (14.0%)	0.649	4 (13.8%)	3 (8.6%)	0.505	0.411
Positive culture for MTB	30 (75.0%)	N/A	N/A	25 (86.2%)	N/A	N/A	N/A
Positive GeneXpert MTB/RIF	31 (77.5%)	N/A	N/A	22 (75.9%)	N/A	N/A	N/A

*ATB, active tuberculosis; LTBI, latent tuberculosis infection; MTB, Mycobacterium tuberculosis; N/A, not applicable. *Comparisons were performed between ATB and LTBI groups using Mann-Whitney U test, Chi-square test, or Fisher's exact test. †Comparisons were performed between Qiaokou and Caidian cohorts using Mann-Whitney U test, Chi-square test, or Fisher's exact test. ‡Patients who underwent chemotherapy or took immunosuppressants within 3 months. Data were presented as means ± SD or numbers (percentages).*

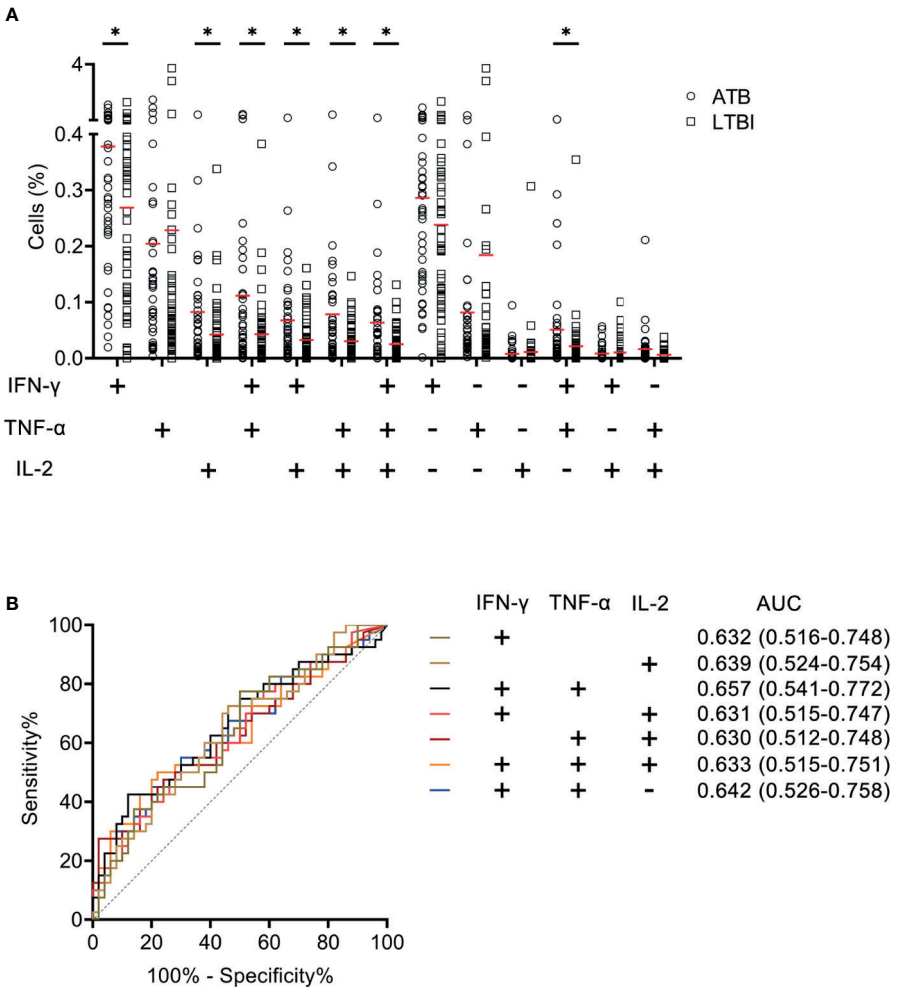

FIGURE 2 | The performance of various cytokine expression pattern upon MTB antigen stimulation in distinguishing ATB patients from LTBI individuals in Qiaokou cohort. **(A)** Aligned dot plots showing the results of the expression pattern of IFN-γ, TNF-α, and IL-2 upon MTB antigen stimulation in ATB patients and LTBI individuals. Horizontal lines indicate the medians. **(B)** ROC analysis showing the performance of various cytokine expression pattern in discriminating ATB patients from LTBI individuals. *$P < 0.05$ (Mann-Whitney U test). MTB, *Mycobacterium tuberculosis*; ATB, active tuberculosis; LTBI, latent tuberculosis infection; AUC, area under the curve.

were 0.699 (95% CI, 0.589–0.809) for CD25 and 0.625 (95% CI, 0.508–0.742) for CD38 (**Figure 3B**). When combining with CD27, no improved performance was obtained for the purpose of distinguishing ATB patients from LTBI individuals (**Figure 3C**).

Activation Markers on MTB-Specific Cells for Differentiating ATB From LTBI

We examined the expression levels of activation markers on MTB-specific cells determined by Th1 cytokine expression. Obvious difference was found in HLA-DR and CD38 expression on MTB-specific cells between ATB patients and LTBI individuals, while no significant difference was observed in CD69 and CD25 expression between these two groups (**Figure 4**). ATB patients showed significantly higher expression of HLA-DR and CD38, especially on polyfunctional MTB-specific cells, compared to LTBI individuals (**Figures 4A, C**). Specially, when a threshold was set as 44%, HLA-DR on TNF-α^+IL-2^+ cells was able to discriminate ATB patients from LTBI individuals with a sensitivity of 93.75% (95% CI, 79.85–98.27%) and specificity of 72.97% (95% CI, 57.02–84.60%) (**Table 2** and **Figure 4B**). The comparison between AUCs showed that the performance of HLA-DR on TNF-α^+ cells was superior to that on IFN-γ^+ cells (Z test, $P < 0.05$). Moreover, HLA-DR on IFN-γ^+TNF-α^+, IFN-γ^+IL-2^+, and TNF-α^+IL-2^+ cells had superiority over that on IFN-γ^+ cells in differentiating ATB from LTBI (Z test, $P < 0.05$). Meanwhile, it was observable that CD27 expression on MTB-specific cells was significantly lower in ATB patients to that in LTBI individuals (**Figures 4G, H**). The value of combination of activation markers and CD27 was also analyzed for differential diagnosis purpose. However, there was no added benefit observed (**Supplementary Figure S1**).

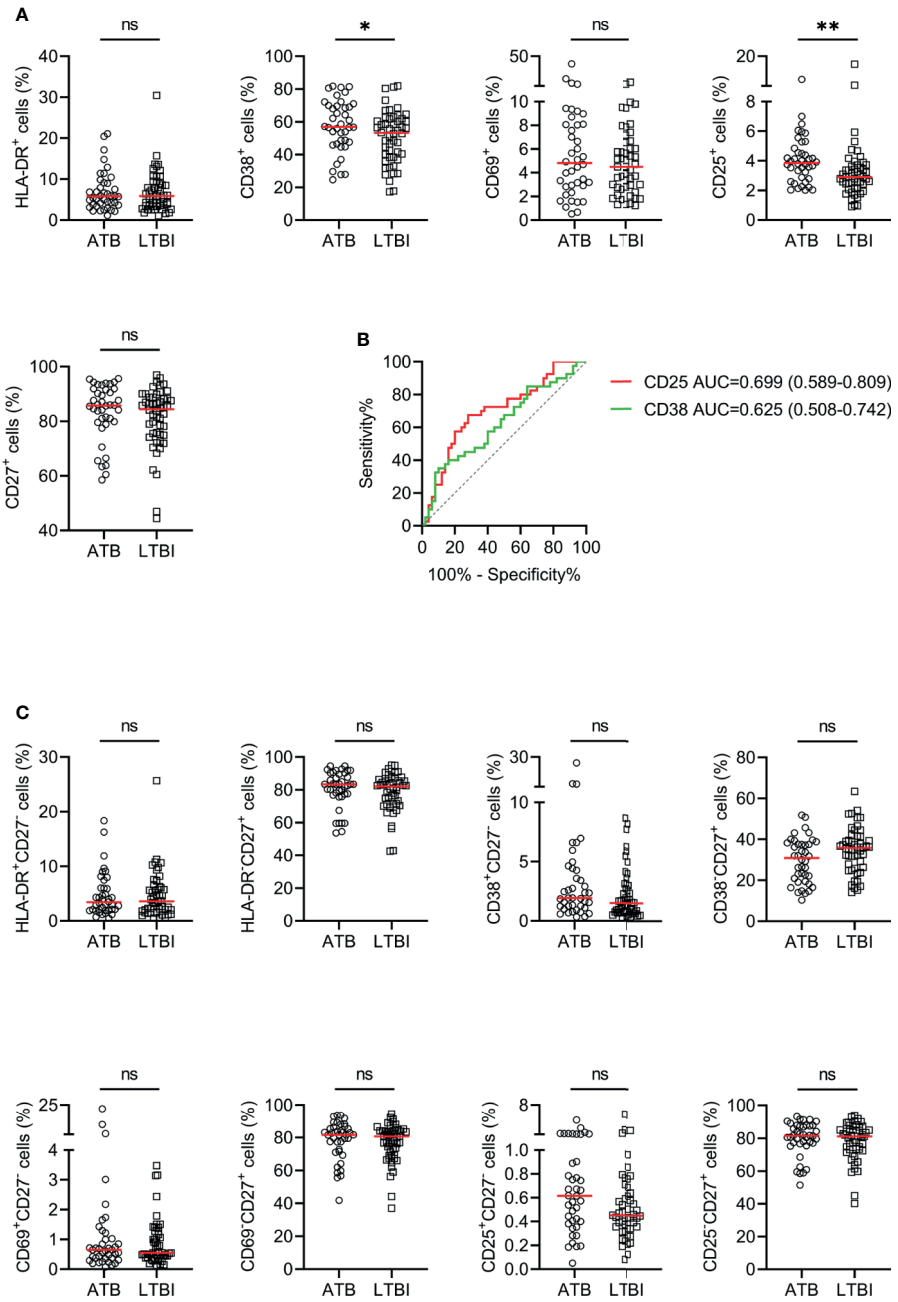

FIGURE 3 | The performance of various markers on $CD4^+$ T cells in distinguishing ATB patients from LTBI individuals in Qiaokou cohort. **(A)** Scatter dot plots showing the results of the expression of HLA-DR, CD38, CD69, CD25, and CD27 on $CD4^+$ T cells in ATB patients and LTBI individuals. Horizontal lines indicate the medians. **(B)** ROC analysis showing the performance of the expression of CD38 and CD25 on $CD4^+$ T cells in discriminating ATB patients from LTBI individuals. **(C)** Scatter dot plots showing the results of the proportions of $HLA\text{-}DR^+CD27^-$ cells, $HLA\text{-}DR^-CD27^+$ cells, $CD38^+CD27^-$ cells, $CD38^-CD27^+$ cells, $CD25^+CD27^-$ cells, and $CD25^-CD27^+$ cells of $CD4^+$ T cells in ATB patients and LTBI individuals. Horizontal lines indicate the medians. $^*P < 0.05$, $^{**}P < 0.01$, ns, no significance (Mann-Whitney *U* test). MTB, *Mycobacterium tuberculosis*; ATB, active tuberculosis; LTBI, latent tuberculosis infection; AUC, area under the curve.

Validation of Activation Markers on MTB-Specific Cells for Differential Diagnosis Between ATB and LTBI

Another blinded set (Caidian cohort) was enrolled to validate the performance of biomarkers discovered in Qiaokou cohort. Excellent performance of HLA-DR on polyfunctional MTB-specific cells was found for the differential diagnosis between ATB patients and LTBI individuals. With the threshold of 48%, the sensitivity and specificity of HLA-DR on IFN-γ^+TNF-α^+ cells were 82.61% (95% CI, 62.86–93.02%) and 69.70% (95% CI, 52.66–82.63%), respectively (**Table 3**,

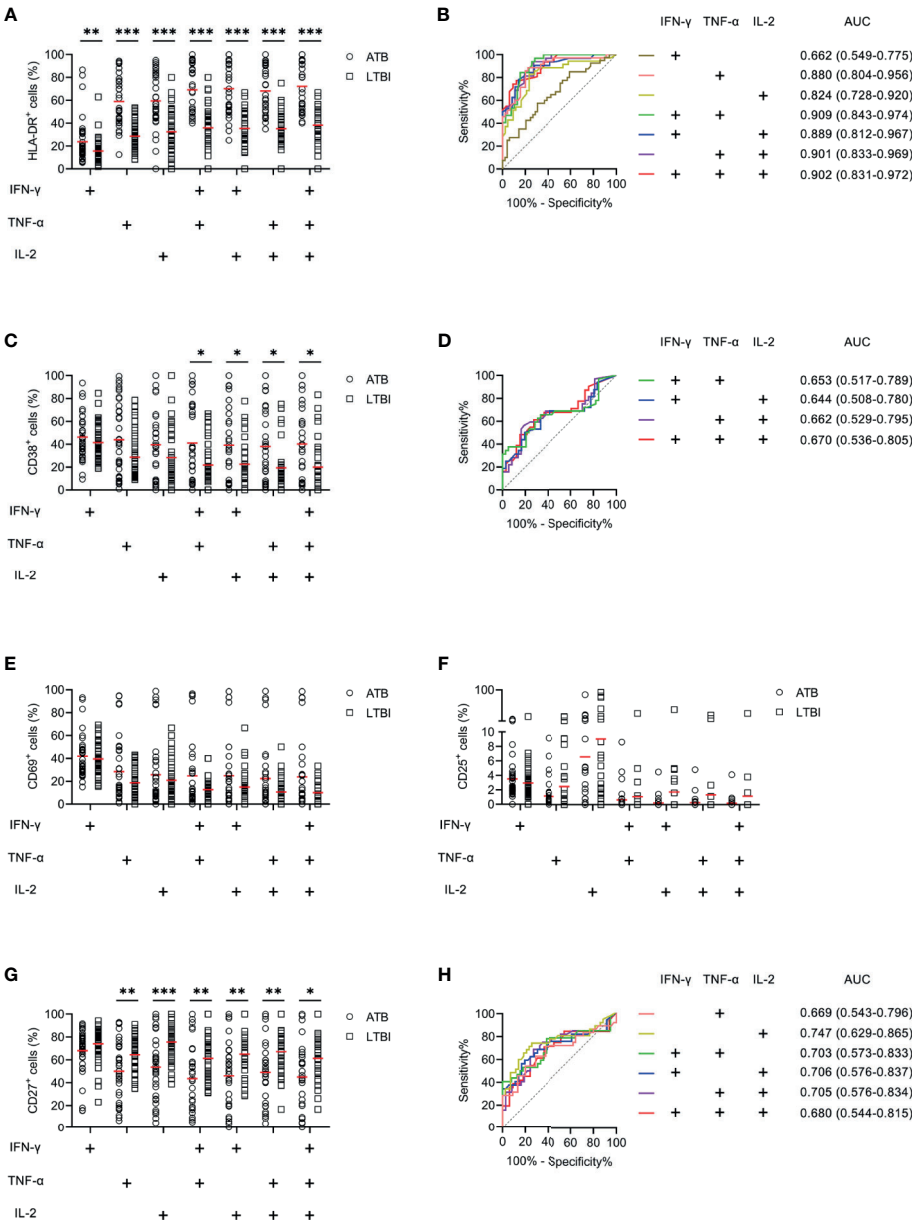

FIGURE 4 | The performance of activation markers on MTB-specific CD4$^+$ T cells in distinguishing ATB patients from LTBI individuals in Qiaokou cohort. **(A)** Aligned dot plots showing the results of HLA-DR expression on MTB-specific cells in ATB patients and LTBI individuals. Horizontal lines indicate the medians. **(B)** ROC analysis showing the performance of HLA-DR expression on MTB-specific cells in discriminating ATB patients from LTBI individuals. **(C)** Aligned dot plots showing the results of CD38 expression on MTB-specific cells in ATB patients and LTBI individuals. Horizontal lines indicate the medians. **(D)** ROC analysis showing the performance of CD38 expression on MTB-specific cells in discriminating ATB patients from LTBI individuals. **(E)** Aligned dot plots showing the results of CD69 expression on MTB-specific cells in ATB patients and LTBI individuals. Horizontal lines indicate the medians. **(F)** Aligned dot plots showing the results of CD25 expression on MTB-specific cells in ATB patients and LTBI individuals. Horizontal lines indicate the medians. **(G)** Aligned dot plots showing the results of CD27 expression on MTB-specific cells in ATB patients and LTBI individuals. Horizontal lines indicate the medians. **(H)** ROC analysis showing the performance of CD27 expression on MTB-specific cells in discriminating ATB patients from LTBI individuals. *P < 0.05, **P < 0.01, ***P < 0.001 (Mann-Whitney U test). MTB, *Mycobacterium tuberculosis*; ATB, active tuberculosis; LTBI, latent tuberculosis infection; AUC, area under the curve.

Figures 5A, B). Using a cut-off value of 51%, HLA-DR on IFN-γ^+IL-2^+ cells was able to discriminate between ATB patients and LTBI individuals with a specificity, sensitivity, and AUC of 83.33% (95% CI, 66.44–92.67%), 90.48% (95% CI, 71.09–97.35%), and 0.938 (95% CI, 0.876–1.000), respectively (**Table 3** and **Figures 5A, B**). When a cut-off value of 44% was used, a sensitivity of 90.91% (95% CI, 72.19–97.47%) and a specificity of 68.97% (95% CI, 50.77–82.73%) were observed in HLA-DR on TNF-α^+IL-2^+ cells (**Table 3** and

TABLE 2 | The performance of HLA-DR on MTB-specific cells for distinguishing between ATB and LTBI in Qiaokou cohort.

Variables	Cutoff value	AUC (95% CI)	Sensitivity (95% CI)	Specificity (95% CI)	PPV (95% CI)	NPV (95% CI)	PLR (95% CI)	NLR (95% CI)	Accuracy
HLA-DR on IFN-γ^+ cells (%)	17	0.662 (0.549–0.775)	57.50% (42.19–71.49%)	65.31% (51.31–77.08%)	57.50% (42.19–71.49%)	65.31% (51.31–77.08%)	1.66 (1.04–2.65)	0.65 (0.43–0.98)	61.80%
HLA-DR on TNF-α^+ cells (%)	38	0.880 (0.804–0.956)	85.71% (70.63–93.74%)	77.78% (63.73–87.46%)	75.00% (59.81–85.81%)	87.50% (73.89–94.54%)	3.86 (2.2–6.77)	0.18 (0.08–0.42)	81.25%
HLA-DR on IL-2$^+$ cells (%)	43	0.824 (0.728–0.920)	82.86% (67.32–91.90%)	74.42% (59.76–85.07%)	72.50% (57.17–83.89%)	84.21% (69.58–92.56%)	3.24 (1.9–5.51)	0.23 (0.11–0.49)	78.21%
HLA-DR on IFN-γ^+TNF-α^+ cells (%)	48	0.909 (0.843–0.974)	84.38% (68.25–93.14%)	82.05% (67.33–91.02%)	79.41% (63.20–89.65%)	86.49% (72.02–94.09%)	4.7 (2.36–9.35)	0.19 (0.08–0.43)	83.10%
HLA-DR on IFN-γ^+IL-2$^+$ cells (%)	51	0.889 (0.812–0.967)	75.00% (57.89–86.75%)	86.11% (71.34–93.92%)	82.76% (65.45–92.40%)	79.49% (64.47–89.22%)	5.40 (2.34–12.48)	0.29 (0.16–0.54)	80.88%
HLA-DR on TNF-α^+IL-2$^+$ cells (%)	44	0.901 (0.833–0.969)	93.75% (79.85–98.27%)	72.97% (57.02–84.60%)	75.00% (59.81–85.81%)	93.10% (78.04–98.09%)	3.47 (2.03–5.93)	0.09 (0.02–0.33)	82.61%
HLA-DR on IFN-γ^+TNF-α^+IL-2$^+$ cells (%)	51	0.902 (0.831–0.972)	77.42% (60.19–88.61%)	84.85% (69.08–93.35%)	82.76% (65.45–92.40%)	80.00% (64.11–89.96%)	5.11 (2.23–11.71)	0.27 (0.14–0.52)	81.25%

MTB, Mycobacterium tuberculosis; ATB, active tuberculosis; LTBI, latent tuberculosis infection; AUC, area under the curve; PPV, positive predictive value; NPV, negative predictive value; PLR, positive likelihood ratio; NLR, negative likelihood ratio; CI, confidence interval.

Figures 5A, B). Furthermore, if using the cut-off value of 51% obtained from Qiaokou cohort, the sensitivity and specificity of HLA-DR on IFN-γ^+TNF-α^+IL-2$^+$ cells were 95.00% (95% CI, 76.39–99.11%) and 82.14% (95% CI, 64.41–92.12%), respectively (**Table 3** and **Figures 5A, B**). Meanwhile, we also analyzed the potential value of the combination of HLA-DR and CD27 for TB diagnostic issue. Consistent with Qiaokou cohort, no obvious improvement was observed after combining CD27 (**Figures 5C–F**). **Table 3** summarized diagnostic performance of HLA-DR on MTB-specific cells when applied to the validation cohort.

DISCUSSION

The lack of efficacious diagnostic tools poses a major challenge to control TB efforts (58, 59). Although many advances have been achieved, especially in omics field (60–62), there were some practical limitations for their clinical application, including expensive laboratory facilities and sophisticated operating procedures. Meanwhile, immunodiagnostics has received considerable attention as an alternative for discrimination of MTB infection status in recent years (63–70). Nevertheless, the identified biomarkers including proteins and cytokines in serum or plasma for diagnostic aim may not be specific for TB due to the influence brought by other immune related diseases such as infection and autoimmune diseases (71–77). Thus, an intensified search for suitable host-specific biomarkers targeting TB diagnostic purpose was urgently needed (78).

With the emergence of flow cytometry as a prominent advancement, many researchers detected makers on immune cell surface or intracellular cytokines for diagnosing infectious diseases (79–81). Some previous works showed that the immune phenotype profile was associated with MTB infection status (82). Howbeit, these evaluations might only denote the global response of the host and could not meticulously reflect the

TABLE 3 | The performance of HLA-DR on MTB-specific cells for distinguishing between ATB and LTBI in Caidian cohort.

Variables	Cutoff value	AUC (95% CI)	Sensitivity (95% CI)	Specificity (95% CI)	PPV (95% CI)	NPV (95% CI)	PLR (95% CI)	NLR (95% CI)	Accuracy
HLA-DR on IFN-γ^+ cells (%)	17	0.510 (0.364–0.657)	39.29% (23.56–57.59%)	60.00% (43.57–74.45%)	44.00% (26.66–62.93%)	55.26% (39.71–69.85%)	0.98 (0.53–1.81)	1.01 (0.68–1.51)	50.79%
HLA-DR on TNF-α^+ cells (%)	38	0.824 (0.723–0.924)	76.92% (57.95–88.97%)	65.71% (49.15–79.17%)	62.50% (45.25–77.07%)	79.31% (61.61–90.16%)	2.24 (1.35–3.72)	0.35 (0.17–0.74)	70.49%
HLA-DR on IL-2$^+$ cells (%)	43	0.881 (0.793–0.970)	81.82% (61.48–92.70%)	70.97% (53.41–83.91%)	66.67% (47.82–81.36%)	84.62% (66.47–93.85%)	2.82 (1.57–5.06)	0.26 (0.1–0.64)	75.47%
HLA-DR on IFN-γ^+TNF-α^+ cells (%)	48	0.891 (0.811–0.972)	82.61% (62.86–93.02%)	69.70% (52.66–82.63%)	65.52% (47.35–80.06%)	85.19% (67.52–94.09%)	2.73 (1.57–4.73)	0.25 (0.1–0.63)	75.00%
HLA-DR on IFN-γ^+IL-2$^+$ cells (%)	51	0.938 (0.876–1.000)	90.48% (71.09–97.35%)	83.33% (66.44–92.67%)	79.17% (59.53–90.76%)	92.59% (76.63–97.95%)	5.43 (2.41–12.23)	0.11 (0.03–0.43)	86.27%
HLA-DR on TNF-α^+IL-2$^+$ cells (%)	44	0.892 (0.806–0.978)	90.91% (72.19–97.47%)	68.97% (50.77–82.73%)	68.97% (50.77–82.73%)	90.91% (72.19–97.47%)	2.93 (1.68–5.12)	0.13 (0.03–0.51)	78.43%
HLA-DR on IFN-γ^+TNF-α^+IL-2$^+$ cells (%)	51	0.946 (0.888–1.000)	95.00% (76.39–99.11%)	82.14% (64.41–92.12%)	79.17% (59.53–90.76%)	95.83% (79.76–99.26%)	5.32 (2.39–11.85)	0.06 (0.01–0.41)	87.50%

MTB, Mycobacterium tuberculosis; ATB, active tuberculosis; LTBI, latent tuberculosis infection; AUC, area under the curve; PPV, positive predictive value; NPV, negative predictive value; PLR, positive likelihood ratio; NLR, negative likelihood ratio; CI, confidence interval.

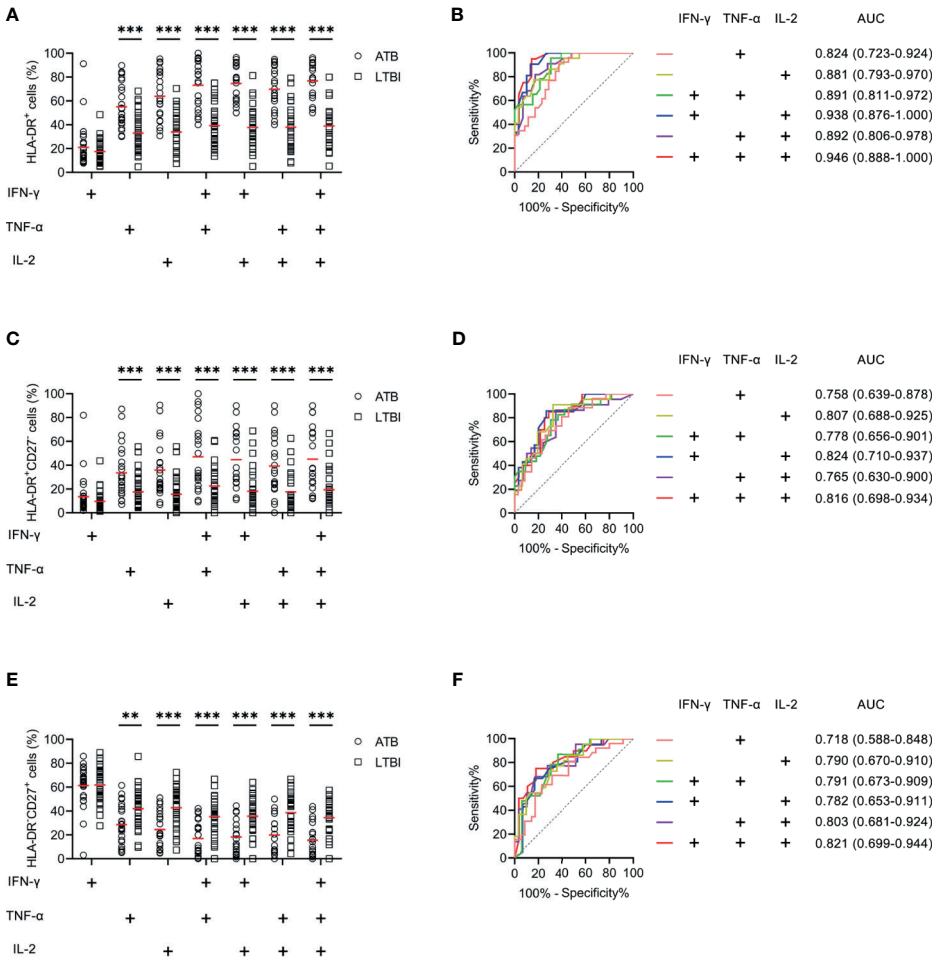

FIGURE 5 | The performance of HLA-DR on MTB-specific cells in distinguishing ATB patients from LTBI individuals in Caidian cohort. **(A)** Aligned dot plots showing HLA-DR expression on MTB-specific cells in ATB patients and LTBI individuals. Horizontal lines indicate the medians. **(B)** ROC analysis showing the performance of HLA-DR expression on MTB-specific cells in discriminating ATB patients from LTBI individuals. **(C)** Aligned dot plots showing the proportions of HLA-DR$^+$CD27$^-$ cells of MTB-specific cells in ATB patients and LTBI individuals. Horizontal lines indicate the medians. **(D)** ROC analysis showing the performance of the proportion of HLA-DR$^+$CD27$^-$ cells of MTB-specific cells in discriminating ATB patients from LTBI individuals. **(E)** Aligned dot plots showing the proportions of HLA-DR$^-$CD27$^+$ cells of MTB-specific cells in ATB patients and LTBI individuals. Horizontal lines indicate the medians. **(F)** ROC analysis showing the performance of the proportion of HLA-DR$^-$CD27$^+$ cells of MTB-specific cells in discriminating ATB patients from LTBI individuals. $^{**}P < 0.01$, $^{***}P < 0.001$ (Mann-Whitney *U* test). MTB, *Mycobacterium tuberculosis*; ATB, active tuberculosis; LTBI, latent tuberculosis infection; AUC, area under the curve.

host-specific immune response to the disease. Thus, the utility of these methods is susceptible to body immunity, and their performance varies greatly in different populations, making it difficult to meet clinical diagnostic need.

Our study focused on MTB-specific cells by gating cells with Th1 cytokine secretion upon MTB antigen stimulation. It is a very small cell subset that can best reflect the host's immune response to MTB infection. We firstly analyzed the cytokine production patterns in ATB patients and LTBI individuals. However, not as reported by previous study (83), no obvious difference was observed. Next, we simultaneously investigated the value of four activation biomarkers including HLA-DR, CD38, CD69, and CD25 on MTB-specific CD4$^+$ T cells, for differentiating ATB patients from LTBI individuals. After ROC curve analysis, two biomarkers of activation (HLA-DR and CD38) showed discriminatory roles. Among them, HLA-DR was the better promising biomarker. Then, we compared the difference of HLA-DR on various MTB-specific cells. Interestingly, we found that the performance of HLA-DR on different MTB-specific cells defined by different cytokine combinations was inconsistent. HLA-DR on TNF-α^+ or IL-2$^+$ cells was remarkably superior to that on IFN-γ^+ cells in distinguishing ATB patients from LTBI individuals. Besides, HLA-DR on polyfunctional MTB-specific cells showed a higher capability than that on MTB-specific cells defined by one cytokine secretion. These data signified the heterogeneity of MTB-specific cells, and the selection of cell subset determined the diagnostic performance of specific biomarkers. Meanwhile,

an opposite trend to HLA-DR was observed in CD27 on MTB-specific cells in ATB patients and LTBI individuals. Thus, we tried to improve the efficacy for discrimination by the combination of HLA-DR with CD27. Notwithstanding, no increased or even decreased performance was obtained. Therefore, independent HLA-DR on MTB-specific cells, rather than combination with others, is more likely to be recommended as a diagnostic biomarker.

Three points should be noted: first, it was observed that HLA-DR on IFN-γ^+ cells had relatively poor performance in distinguishing ATB from LTBI when comparing to that of TNF-α^+ or IL-2^+ cells. We found that the percentages of IFN-γ^+ cells in unstimulated tubes ranged from 0.02% to 0.1%, while the percentages of TNF-α^+ cells and IL-2^+ cells in unstimulated tubes ranged from 0.01% to 0.04%. The background value for IFN-γ was relatively higher than that for TNF-α and IL-2. Thus, some of the IFN-γ^+ cells in stimulated tubes were not MTB-specific cells. It may be one reason for the poor performance of HLA-DR on IFN-γ^+ cells in differentiating ATB from LTBI. Second, the variation of HLA-DR expression in the LTBI group might be due to the different infection status. Some individuals in the LTBI group have been infected for a long time, while the others were infected with MTB recently. As indicated by previous study, some subjects with recent MTB infection would also have high HLA-DR expression on MTB-specific cells (84). Thus, another approach that could be combined with markers in the present study to improve diagnostic specificity should be developed in the future. Third, given that the increasing number of MTB antigen has been identified in recent years and the response patterns of PBMCs varied upon different antigen stimulation (85–87), the investigation targeting optimal antigen selection is needed in the future.

Several limitations should be mentioned. First, although there were two centers in the current study, the number of recruited partakers in each center was circumscribed. These biomarkers should be further validated in larger cohorts. Second, although some diseases including diabetes and tumors were involved in underlying condition of the enrolled patients in this study, further investigation is required to elucidate the influence of other diseases such as COVID-19 on the performance of these biomarkers. Finally, given the fact that the biomarkers detected in the present study were on MTB-specific cells, HLA-DR would be useless when applying to cases with few MTB-specific cells, such as T-SPOT-negative individuals (88, 89). Hence, more reasonable methods for this population should be developed in the future.

In conclusion, our study demonstrated that HLA-DR on MTB-specific cells has robust diagnostic potential for discrimination between ATB and LTBI. Notably, the detection of biomarkers discovered in the present study is amenable to the existing platforms.

DATA AVAILABILITY STATEMENT

The original contributions presented in the study are included in the article/**Supplementary Material**. Further inquiries can be directed to the corresponding authors.

ETHICS STATEMENT

The studies involving human participants were reviewed and approved by the committee of Tongji hospital, Tongji Medical College, Huazhong University of Science and Technology. The patients/participants provided their written informed consent to participate in this study.

AUTHOR CONTRIBUTIONS

YL designed the study. LM, QL, and GT set up the clinical cohorts at the respective hospitals. HS, LW, ST, HH, MH, and RO included patients and collected data. YL performed the main experiment. YL analyzed and interpreted the data. YL and YX did the statistical analysis. YL draft the manuscript. YL, FW, and ZS contributed to the revision of manuscript. All authors contributed to the article and approved the submitted version.

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SUPPLEMENTARY MATERIAL

The Supplementary Material for this article can be found online at: https://www.frontiersin.org/articles/10.3389/fimmu.2021.721013/full#supplementary-material

Supplementary Figure 1 | The performance of the combination of activation markers and CD27 on MTB-specific cells in distinguishing ATB patients from LTBI individuals in Qiaokou cohort. **(A)** Aligned dot plots showing the proportions of HLA-DR^+CD27^- and HLA-DR^-CD27^+ cells of MTB-specific cells in ATB patients and LTBI individuals. Horizontal lines indicate the medians. **(B)** ROC analysis showing the performance of the proportions of HLA-DR^+CD27^- and HLA-DR^-CD27^+ cells of MTB-specific cells in discriminating ATB patients from LTBI individuals. **(C)** Aligned dot plots showing the proportions of $CD38^+CD27^-$ and $CD38^-CD27^+$ cells of MTB-specific cells in ATB patients and LTBI individuals. Horizontal lines indicate the medians. **(D)** ROC analysis showing the performance of the proportions of $CD38^+CD27^-$ and $CD38^-CD27^+$ cells of MTB-specific cells in discriminating ATB patients from LTBI individuals. **(E)** Aligned dot plots showing the proportions of $CD69^+CD27^-$ and $CD69^-CD27^+$ cells of MTB-specific cells in ATB patients and LTBI individuals. Horizontal lines indicate the medians. **(F)** ROC analysis showing the performance of the proportions of $CD69^+CD27^-$ and $CD69^-CD27^+$ cells of MTB-specific cells in discriminating ATB patients from LTBI individuals. **(G)** Aligned dot plots showing the proportions of $CD25^+CD27^-$ and $CD25^-CD27^+$ cells of MTB-

specific cells in ATB patients and LTBI individuals. Horizontal lines indicate the medians. **(H)** ROC analysis showing the performance of the proportions of $CD25^+CD27^-$ and $CD25^-CD27^+$ cells of MTB-specific cells in discriminating ATB patients from LTBI individuals. $^*P < 0.05$, $^{**}P < 0.01$, $^{***}P < 0.001$ (Mann-Whitney *U* test). MTB, *Mycobacterium tuberculosis*; ATB, active tuberculosis; LTBI, latent tuberculosis infection; AUC, area under the curve.

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A Dual Marker for Monitoring MDR-TB Treatment: Host-Derived miRNAs and *M. tuberculosis*-Derived RNA Sequences in Serum

*Claudia Carranza[1], María Teresa Herrera[2], Silvia Guzmán-Beltrán[2], Manuel Guadalupe Salgado-Cantú[2], Ivan Salido-Guadarrama[2], Elizabeth Santiago[3], Leslie Chávez-Galán[4], Luis Horacio Gutiérrez-González[5] and Yolanda González[2]**

[1] Laboratorio de Inmunobiología de la Tuberculosis, Instituto Nacional de Enfermedades Respiratorias Ismael Cosío Villegas, Mexico, Mexico, [2] Department of Microbiology, National Institute for Respiratory Diseases Ismael Cosío Villegas, Mexico, Mexico, [3] Laboratorio de Biología Computacional, Instituto Nacional de Enfermedades Respiratorias Ismael Cosío Villegas, Mexico, Mexico, [4] Laboratory of Integrative Immunology, Instituto Nacional de Enfermedades Respiratorias Ismael Cosío Villegas, Mexico, Mexico, [5] Department of Virology and Mycology, Instituto Nacional de Enfermedades Respiratorias Ismael Cosío Villegas, Mexico, Mexico

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Novel N. Chegou,
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Roberta Olmo Pinheiro,
Fundação Oswaldo Cruz (Fiocruz), Brazil
Jina Ko,
Massachusetts General Hospital and Harvard Medical School, United States

***Correspondence:**
Yolanda González
ygonzalezh@iner.gob.mx

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Background: In the absence of a late marker of treatment failure or relapse in MDR-TB patients, biomarkers based on host-miRNAs coupled with *M. tuberculosis*-RNAs evaluated in extracellular vesicles (EVs) are an alternative follow-up for MDR-TB disease. Characterization of EVs cargo to identify differentially expressed miRNAs before and after treatment, and to identify *M. tuberculosis*-derived RNA in serum EVs from resistant TB patients.

Methods: EVs were isolated from serum of 26 drug-resistant TB (DR-TB) patients and 16 healthy subjects. Differential expression of miRNAs in pooled exosomes from both untreated and treated patients was assessed and individually validated at different time points during treatment. In addition, *M. tuberculosis* RNA was amplified in the same samples by qPCR.

Results: A multivariate analysis using miR-let-7e-5p, -197-3p and -223-3p were found to be a more sensitive discriminator between healthy individuals and those with TB for both DR-TB (AUC= 0.96, 95%, CI=0.907-1) and MDR-TB groups (AUC= 0.95, 95%, CI= 0.89-1). Upregulation of miR-let-7e-5p were observed at the time of *M. tuberculosis* negative culture T(3-5) for MDR-TB group or for long-term T(9-15) for MDR-TB group without diabetes (T2DM). A second pathogen-based marker based on 30kDa and 5KST sequences was detected in 33% of the MDR-TB patients after the intensive phase of treatment. The miR-let7e-5p is a candidate biomarker for long-term monitoring of treatment for the group of MDR-TB without T2DM. A dual marker of host-derived miR-let7e-5p and *M. tuberculosis*-derived RNA for monitoring-TB treatment based in serum EVs.

Conclusion: A dual marker consisting of host-derived miR-let7e-5p and *M. tuberculosis*-derived RNA, could be an indicator of treatment failure or relapse time after treatment was completed.

Keywords: drug resistance to TB, miRNAs, exosomes, *M. tuberculosis*-RNA, monitoring treatment

INTRODUCTION

Tuberculosis (TB) is a transmissible disease which still is one of the top ten causes of death worldwide. Only in 2019, between 8.9 and 11 million lived with the active TB, and 2.4 million people died from it. One quarter of the world population could already be infected with Mycobacterium tuberculosis (*M. tuberculosis*) and, therefore, at risk of developing the active form of the disease (1).

The risk of TB reactivation increases significantly in people with risk factors such as diabetes mellitus (DM), human immunodeficiency virus (HIV) coinfection, and many other conditions associated with immunocompromise (1).

Currently, treatment of drug-sensitive TB consists of a six-month regimen based on four drugs. However, drug-resistant TB (DR-TB) is defined when a person has drug resistant TB for at least one of the main TB drugs, and multi drug resistant TB (MDR-TB) is defined when a person has drug resistance to rifampicin and isoniazid, the two most efficacious first-line anti-tuberculosis drugs. Both types of drug resistant TB remain as significant challenges and major contributors to mortality. According to the same report from WHO, TB-DR killed 465,000 people in 2019 (1). Drug resistance is more probable in patients non-compliance to treatment or a primary infection with a drug-resistant strain (DR-TB) (2, 3). Treatment for multi-drug resistant tuberculosis (MDR-TB) treatment is longer (two years), more toxic and much less successful (57% cure rate) (1).

Most diagnostic tests for TB are based on either bacteriological culture or polymerase-chain reactions (PCR). Traditionally, sputum smear microscopy has been the most widely used test. Despite its high specificity, its diagnostic value is limited by its very low sensitivity. Cultures, on the other hand, can take up to 42 days to reveal detectable growth (4, 5). The Xpert MTB/RIF and the ultra-version tests detect genetic material from *M. tuberculosis*, with a sensitivity similar to microbial cultures (6). These tests yield quick results but are costly and not widely available (7).

All these diagnostic assays require a positive culture for *M. tuberculosis.* However, sputum samples from individuals with active TB, especially those with type 2 diabetes mellitus (T2DM), often fail to yield bacteria. In these cases, invasive procedures are often needed for diagnosis (8). Thus, sensitive, and specific diagnostic tests that can spare patients from more invasive procedures are warranted.

A negative sputum culture is generally considered a marker of treatment success. Nevertheless, some patients develop recurrent TB despite negative sputum cultures, which may indicate the presence of viable but nonculturable bacteria. Therefore, a reliable marker of recovery or recurrence is clearly needed (9). In many diseases, including TB, biomarkers could be useful in the diagnosis of the disease, in predicting the development of an active form and in the assessment of response to treatment or vaccination (6, 10).

While the biomarkers used for diseases are molecules produced by the host cells, the TB markers consist of mycobacterial antigens, metabolites, transcription signals and proteins that are secreted from *M. tuberculosis* in replicative status (2). Both markers may be found in extracellular vesicles (EVs) secreted by infected cells, which in turn contain microvesicles of about 30 to 100 nm. Microvesicles or exosomes have a complex composition depending on the cell of origin and may be found in body fluids like blood or serum. It has been shown that exosomes secreted by cells are essential for intercellular communication, since they can carry proteins, RNA, and DNA (11–13).

Exosomes released from infected cells can transmit signals and transfer molecules to recipient cells to trigger changes in their physiology (14, 15). In particular, microRNAs (miRNAs) are transported by exosomes which are involved in the pathogenesis of several respiratory diseases, including TB, in which they play a relevant role in pulmonary inflammation and pathogenesis (14, 15). The expression of miRNA varies across different pathological conditions, which suggests that microvesicles may also reflect disease status (16).

It has been observed that *M. tuberculosis*-infected macrophages can secrete miRNAs within exosomes. *In vitro* studies have shown that monocyte-derived macrophages infected by *Mycobacterium bovis* BCG secrete exosomes containing miR-1224, -1293, -425, -4467, -4732, -484, -5094, -6848, -6849, -4488, and -96 (17).

By the same token, exosomes miRNAs of individuals with TB infection have been compared with those healthy volunteers (18). The miR-1246, -2110, -370-3P, -28-3p and -193b-5p are expressed in those with active TB. Furthermore, exosomes may contain mycobacterial miRNA as well as highly antigenic mycobacterial proteins such as ESAT-6 (Rv3875), the Ag85 complex (Rv3804c, Rv1886c, and Rv0129c), MPT64 (1980c), and MPT63 (1926c) (19).

Since there seems to be a TB-specific pattern of exosomes miRNA secretion, it follows that these molecules can be potential biomarkers of DR-TB. To prove this, more studies are needed to evaluate exosomes miRNAs in actual patients with DR-TB. Knowledge generated from such studies could help in the diagnosis, prognosis, and treatment of the disease. Thereby, in this study we identify the spectrum of miRNAs generated in host's lung tissue in response to *M. tuberculosis* and sequences from mycobacteria that circulate within exosomes. This will help us to identify those markers associated to the pathogen as well as those associated with the host.

METHODS

Study Participants

Twenty-six patients with DR-TB were studied between 2010 and 2018. DR-TB group included those with rifampicin resistant (RR), multi-drug resistant (MDR), and extensively drug-resistant (XDR) strains; all patients had a confirmed diagnosis of TB and were under Directly Observed Therapy (DOT). Patients were followed for 15 months at the Instituto Nacional de Enfermedades Respiratorias (INER) in Mexico City. A blood sample was taken at each follow-up visit. This sample was centrifuged to obtain serum and stored at -80°C. Additionally, 16 healthy volunteers were included as control group and their samples underwent the same procedure. An informed consent was obtained from all participants. The use of these samples was approved by the Institutional Review Board. Written consent was obtained from all participants, and the Ethics Committee approved this study (No. C57-17).

Biological Samples Tested

We analyzed the samples from 26 TB patients with drug resistant TB diagnoses (21 MDR, three RR and two XDR); the blood samples were taken at: T(0), untreated patients; T(3-5), one sample between 3 to 5 months after treatment; T(6-8), one sample between 6 to 8 months after treatment, and T(9-15), one sample between 9 to 15 months after treatment. A total of four samples per patient were processed. The samples were classified into four categories: 1) those from DR-TB patients exhibiting any resistance types (RR, MDR or XDR); 2) those from MDR-TB patients; 3) those from MDR-TB patients without T2DM (MDR-TB-T2DM−, and 4) those from MDR-TB patients with T2DM (MDR-TB-T2DM+).

Exosomes Extraction From Serum

Exosomes were purified from the serum using the Exo-Quick™ exosome precipitation solution (System Biosciences, USA), following the instructions of the manufacturer, and adding a filtration procedure step with a 0.22 µm filter before the procedure. In brief, filter serum was centrifuged at 3000 g and ExoQuick™ exosome precipitation solution was added and incubated, then it was centrifuged at 1500 g and the pellet was resuspended in 200 µl of PBS. Exosomes or EVs were obtained from 300 µl of serum to perform DNA and RNA extraction, protein extraction and miRNA analysis. EVs were stored at -20°C until further processing.

Extraction and Quantification of Exosomes Proteins

Protein was obtained from 100 µl of exosomes using buffer RIPA (cat. 89901, Fisher Scientific, USA.) plus a protease inhibitor cocktail (cat. 78430, Thermo Fisher Scientific, USA). The mix was sonicated for 15 seconds for total lysis and then centrifuged at 14000 g for 15 minutes at 4°C. The supernatant was recovered, and total protein was quantified using the Quick Start assay (cat. 500-0202, Bio-Rad, USA). The absorbance was read at 595 nm (Epoch™, Bioteck) and the protein quantitation was calculated by standard curve.

SDS-PAGE-Western Blot Assay for Exosomal Tetraspanin and Exosomal *M. tuberculosis*-Derivate Proteins

The EVs total protein was mixed with Laemmli buffer (cat.1610747, Bio-Rad, USA) with β-mercaptoethanol (Bio-Rad, USA). Then, proteins were separated by SDS-polyacrylamide gel and transferred to a nitrocellulose membrane Immobilon-P (MilliporeSigma, USA). The membranes were incubated with anti-CD63 (ab216130, Abcam, UK), -CD9, -CD81, -hsp70 ExoAb Antibody Kit (cat. EXOAB kit, System Biosciences, USA), ESAT-6 (ab26246, Abcam, U.K), -CFP-10 (PA1-19445, Thermo Fisher, USA), -Ag-38kDa (NB100-62769, Novus Biological, USA), -Ag-85B (ab43019, Abcam, U.K), and -MPT64 (CSB-PA14947A0Rb, Cusabio, USA), and then with anti-IgG-HRP (cat. EXOAB kit) or anti-IgG-HRP (65-6120, Thermo Fisher, USA). The chemiluminescent substrate Clarity (cat. 170-5060, Bio-Rad, CA, USA) was added, and immunocomplexes were detected using system ChemiDocMP Imaging (Bio-Rad, Hercules, USA). CD63 and CD9 tatraspanins expression is shown in the workflow chart (**Figure 1**).

Extraction of Total Small RNAs and miRNA Retrotranscription From Serum Exosomes

The RNA was obtained with the MagMAX™ *mir*Vana™ Total RNA Isolation Kit (cat. A27828, Applied Biosystems-Thermo Fisher Scientific, USA), which was used as per the instructions of the manufacturer. The total RNA of exosomes was recovered and stored at -20°C until further use.

For RNA synthesis of complementary DNA was used the TaqMan™ Advanced miRNA cDNA Synthesis Kit (cat. A28007, Applied Biosystems-Thermo Fisher Scientific, USA) following the manufacturer instructions. RNA-samples were thawed and underwent three procedures in sequence: first a polyadenylation of miRNA catalyzed by one poliA polymerase, then an adaptive ligation and finally a retrotranscription by an inverse transcriptase. The complementary DNA (cDNA) was synthetized in the Step One Plus instrument (Applied Biosystems, Thermo Fisher Scientific, USA), according to manufacturer instructions and stored at -20°C until further use.

Evaluation of Endogenous miRNAs Expression From Exploratory Array

The miRNA Human Endogenous Controls 96-well Plate TaqMan™ Advanced (cat. A34642, Applied Biosystems-Thermo Fisher Scientific, USA) was used to evaluate 30 endogenous control miRNAs, including two exogenous (non-human) controls for normalization of sample input. Briefly, PCR was performed mix the TaqMan Fast Advanced Master Mix (cat. A44360 Applied Biosystems-Thermo Fisher Scientific, USA) and the pool of cDNA from five MDR-TB patients before treatment (T0) or after 12 months of treatment (T12) and the mix was added to plates. The qPCR was run in the Step One Plus instrument according to manufacturer instructions. The data are shown in the **Supplementary File**.

Evaluation of Exosome miRNAs Expression From Exploratory Array and Individual Validation

We used pre-spotted TaqMan Advanced miRNA assays in a 96-well fast plate (cat. A31813 Applied Biosystems, Thermo Fisher Scientific, USA). This kit contains two plates for 188 unique miRNAs for serum/plasma samples, 2 exogenous controls (cel-miR-39-3p, ath-miR159a) and an endogenous control (hsa-miR-16-5p) for normalization of data results. Briefly, PCR was performed by mixing the TaqMan Fast Advanced Master Mix solution and the pool of cDNA from five MDR-TB patients at T(0) or T(12), and added to plates. The qPCR was run in the Step One Plus instrument, according to manufacturer instructions. The differentially expressed miRNAs in the pool at T(0) *vs* T(12) are shown in the workflow (**Figure 1**) and in the **Supplementary File**.

For individual validation, we selected eight miRNAs: two endogenously expressed at C_T<25, three miRNAs expressed at T (0) and down-expressing after 12 months with C_T<32, and three miRNAs not expressed at T(0) and up-expressed at T(12) with C_T<32. Briefly, cDNA for individual miRNAs expression was evaluated at four different times: T(0), T (3-5), T(5-8) and T(9-15), when it was mixed with the TaqMan Fast Advanced Master Mix and the specific probe for microRNA assays (Custom TaqMan®. Applied Biosystems, ThermoFisher Scientific, CA, USA), according to the below table.

miRNA name	Target sequence
hsa-miR-20a-3p	ACUGCAUUAUGAGCACUUAAAG
hsa-miR-195-5p	UAGCAGCACAGAAAUAUUGGC
hsa-miR-328-3p	CUGGCCCUCUCUGCCCUUCCGU
hsa-miR-145-5p	GUCCAGUUUUCCCAGGAAUCCCU
hsa-let-7e-5p	UGAGGUAGGAGGUUGUAUAGUU
hsa-miR-197-3p	UUCACCACCUUCUCCACCCAGC
hsa-miR-223-3p	UGUCAGUUUGUCAAAUACCCCA
hsa-miR-451a	AAACCGUUACCAUUACUGAGUU

The qPCR was run in the Step One Plus instrument, according to manufacturer instructions. The C_T values of individual miRNAs are shown in the **Supplementary File**.

DNA and Total RNA Exosome Extraction and *M. tuberculosis* Sequences Amplification by qPCR

The DNA was isolated with the Exosomal DNA Extraction Kit (101Bio, USA) according to the manufacturer instructions. RNA was extracted with the total exosome RNA and protein isolation kit (Invitrogen-ThermoFisher Scientific, USA) according to the manufacturer instructions. Then, the complementary DNA (cDNA) was synthetized using RNA as template and the TaqMan™ MicroRNA Reverse Transcription kit (cat. 4366597, Applied Biosystem-Thermo Fisher Scientific, USA) with hexamers (cat. N8080127, Invitrogen, Thermo Fisher Scientific Inc., USA), and was amplified using a thermocycler Verity (Applied Biosystems-ThermoFisher Scientific, USA)

Finally, mycobacterial sequences were evaluated by quantitative real time PCR (qPCR), using exosomal cDNA, Maxima SYBR™ Green/ROX qPCR Master Mix (Thermo Scientific Inc., USA), and the specific forward/reverse primers for *M. tuberculosis* antigens.

Antigen	Primers 5´- 3´
MPT64	FW: GTGAACTGAGCAAGCAGACCG
	RV: GTTCTGATAATTCACCGGGTCC
MPB64	FW: GACTTCTGGTCGGGGTAGTAAC
	RV: CTGTCGTTTTGCTCTGTTGTTC
5KST	FW: TTGCTGAACTTGACCTGCCCGTA
	RV: GCGTCTCTGCCTTCCTCCGAT
19 kDA	FW: GAGACCACGACCGCGGCAGG
	RV: AATGCCGGTCGCCGCCCCGCCGAT
30 kDa	FW: TGTACCAGTCGCTGTAGAAG
	RV: GACATCAAGGTTCAGTTCC
CFP-10	FW: AGGTAATTTCGAGCGGAT
	RV: CACTGGCCCTGCAACGAA
ESAT-6	FW: AAGCTCGCAGCGGCCTGG
	RV: CCTGACCGGCTTCGCTGA
Ag85c	FW: AAGGTCCAGTTCCAGGGCG
	RV: ATTGGCCGCCCACGGGCATGAT
16S rRNA	FW: GCCGTAAACGGTGGGTACTA
	RV: TGCATGTCAAACCCAGGTAA

The DNA from *M. tuberculosis* H37Rv strain was included as positive control and water for PCR was used as a negative control. In the samples, a result was considered positive when the melting temperature was comparable to the positive control. Then, we visualized the amplicons in agarose gel electrophoresis (1.8%) with GelRed (cat. 41003, Bioutum) and documented by the ChemiDocMP Imaging System.

Statistical Analysis

Raw C_T data were normalized using the Delta C_T method (δC_T). To that end, average expression stability (defined as M-value) of the reference miRNA was determined using the GeNorm algorithm (20). Briefly, this method iteratively compares the mean expression value of tested miRNAs and selects those with lowest M-value. Then, for each sample we subtracted the C_T value of the most stable miRNA from the C_T value of the remaining miRNAs.

Expression differences between cases and controls were assessed by an unpaired two-samples Mann-Whitney test. To evaluate changes in expression that are associated to treatment, expression at different endpoints (i.e., T(3-5), T(6-9) and T(9-15) months) were compared against expression at T(0). Significance was evaluated with a Wilcoxon signed-rank test and a p-value < 0.05 was considered significant. Receiver Operating Characteristics (ROC) analysis and multiple regression were performed with the pROC package and the glm function in R, respectively. The evaluation of transitions was based on the δC_T value between all the possible time points for all samples of study. The analysis was done using the Matlab R2017b software.

RESULTS

Characteristics of Study Groups

The patients with drug resistant TB included patients with rifampicin resistant (RR), multi-drug resistant (MDR), and

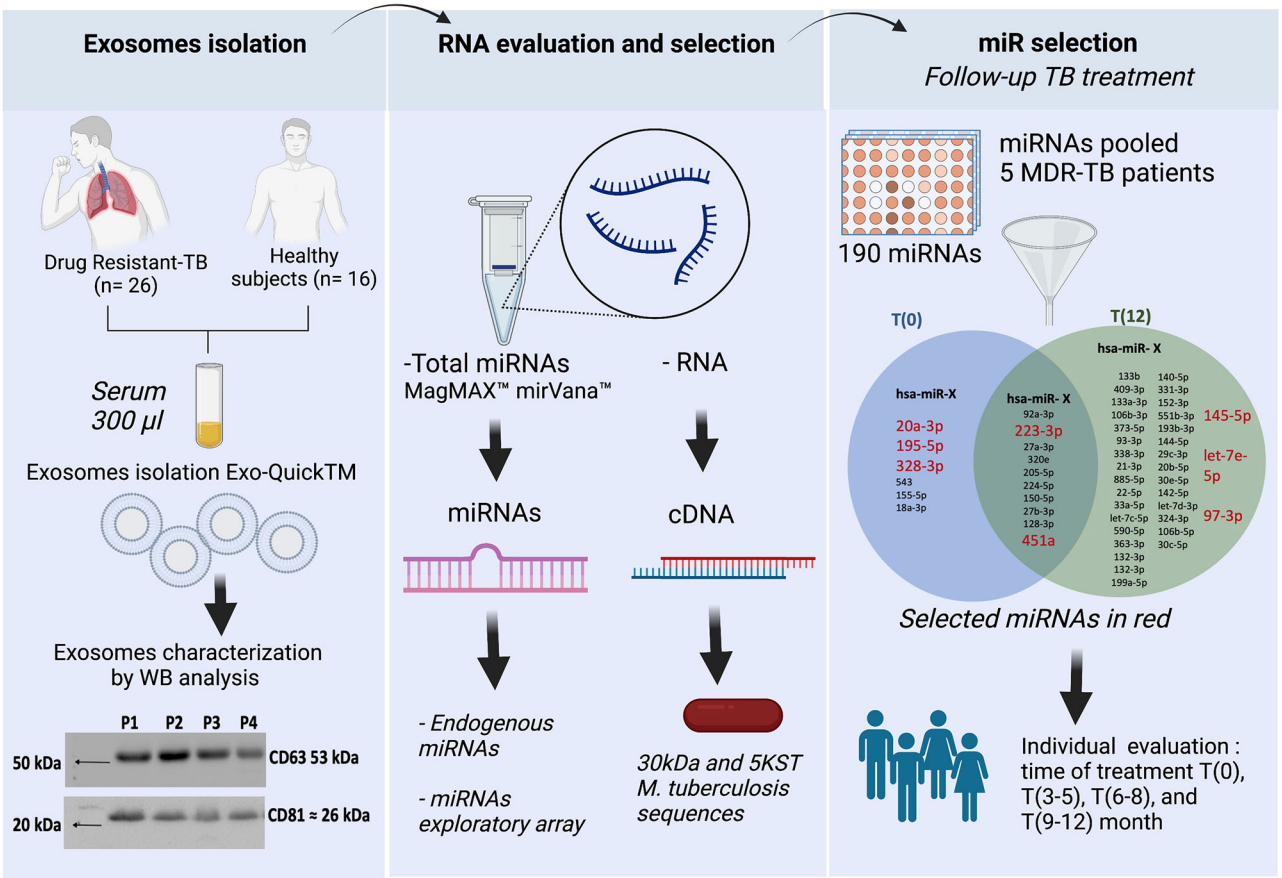


FIGURE 1 | Workflow chart. General strategy in three steps. First step, the exosomes isolation and characterization showed CD63 and CD81 tetraspanin expression in serum exosomes from DR-TB patients. Second step, extraction of both host-miRNAs and *M. tuberculosis*-RNA. Third step, miRNAs differentially expressed in serum exosomes from MDR-TB patients at T(0) and T(12). In red, the 8 miRNAs selected for individual analysis. BioRender, publication and licensing rights (QX22QHOTQ4).

extensively drug-resistant (XDR) strains; all patients had a confirmed diagnosis of TB and were under Directly Observed Therapy (DOT). The 14% of patients were underweight. The main comorbidity was type 2 diabetes mellitus (T2DM), 33% of patients have it. Sixteen age- and sex-matched healthy subjects were included for miRNAs expression analysis (**Table 1**).

Forty miRNAs Differentially Expressed Before and at 12-Month Follow-Up Treatment Were Identified in the Pool of Exosomal miRNAs

There are no constitutive genes for the evaluation of miRNAs; therefore, endogenous genes are used to perform normalization of miRNA expression. The analysis of 32 endogenous miRNAs in a pool of serum exosomes from five MDR-TB patients comparing T(0) *vs* T(12) did not show any stable endogenous miRNAs at any of these time points (**Supplementary File**). However, the analysis of the expression of an array of 180 miRNAs in the same pool of miRNAs showed 10 stable miRNAs in the serum exosome pool at T(0) and (T12), and we selected two of them to normalize the data. Additionally, we identified 40 miRNAs up- or down-expressed at T(0) *vs* T(12). Six were up-expressed at T(0) and 34 were up-expressed at T (12) (**Figure 1**).

Of the 40 miRNAs, we analyzed eight individually: miR-223-3p and -451a, -20a-3p, -195-5p and -328-5p, -145-5p, -197-3p and -let7e-5p. We identified miR145-5p as the miRNA that is more consistently expressed during TB treatment. Therefore, we used it to calculate the ΔC_T. The miRNAs expression from individual serum exosomes are shown in the **Supplementary File**.

Differential Expression of miR-197-3p, miR-223-3p and miR-let7e-5p in DR-TB Disease

The molecular testing shows 69% sensitivity for smear-negative, culture-positive specimina (21). Since some patients with suspected TB cannot be diagnosed through these methods, here we analyzed miRNA expression associated to DR-TB diagnosis.

TABLE 1 | Characteristics of the study participants.

	Patients (n=26)	*Healthy controls (n=15)*	*P value*
Age, yr	43.5 (19–77)	45 (27–61)	0.6859
Sex, male (%)	18 (69.2)	10 (66.6)	>0.9999
BMI	23.2 (17-27)	26 (23-33)	0.0050
Comorbidities			
Diabetes (%)	9 (34.5)	–	–
Alcoholism (%)	9 (34.5)	–	–
Smoking (%)	2 (7.7)	–	–
Malnutrition (%)	15 (5.77)	–	–
Pulmonary Tuberculosis			
RR/MDR-TB (%)	3 (15.4)	–	–
MDR-TB (%)	21 (73)	–	–
XDR-TB (%)	2 (7.7)	–	–
TB Treatment			
Individualized *(%)	9 (42.8)	–	–
Clinical data			
LeukocytesWBC count, $x10^3/mm^3$	9.7 (4.3–18.8)		
Neutrophil count, $x10^3/mm^3$	7.4 (1.7-17.3)		
Lymphocyte count, $x10^3/mm^3$	1.6 (0.9–2.4)		
Hematocrit %	41.2 (27.2-52.8)		
Hemoglobin g/ml	13.6 (9.4-17.7)		
NLR ratio	4.8 (0.9-18.8)		
Metabolic characteristics			
HbA1c % T2DM	9.2 (5.5-13.5)		
Glucose, mg/dl	99 (75-525)		
Blood Chemistry Profile			
Creatinine, mg/dl	0.76 (0.51-1.1)		
BUN, mg/dl	10 (5.8-20)		
Albumin, mg/dl	3.6 (2.3-4.4)		
Urea, mg/dl	22 (12.4-42)		
Renal function			
MDRD ml/min/1.73 m^2	109.3 (67-7167.3)		
CKD/EPI ml/min	104 (64-134)		
CrCL ml/min	102.3 (49-209.2)		
Nephrotoxicity	17.4%		
Hearing damage	35%		

**Individualized include the drugs: standard antibiotics and others such as amoxicillin, clavulanic acid, ceftriaxone, linezolid, clofazimine and dalamanid. BMI, Body Mass Index; RR/MDR-TB, Rifampicin-Resistant-MDR TB; MDR-TB, Multidrug-resistant TB; XDR-TB, Extensively drug-resistant TB; WBC, White Blood Cell; NLR, Neutrophil-Lymphocyte Ratio; HbA1c, hemoglobin A1c; BUN, Blood Urea Nitrogen; MDRD, Modification of Diet in Renal Disease; CKD/EPI, Chronic Kidney Disease Epidemiology Collaboration; CrCL, Creatinine clearance rate.*

We identified three miRNAs associated to DR-TB disease: miR-197-3p and -223-3p, which are downregulated, and miR-let7e-5p, which is upregulated in DR-TB patients compared to healthy subjects (**Figure 2A**). Since the most represented group was that of MDR-TB, we focused our search for differential miRNA expressions in this group, only to find that the same statistical differences were conserved (**Figure 2B**).

miRNA-Model Increases the Sensitivity and Specificity for TB Disease

We calculated the sensitivity and specificity of miR-197-3p, -223-3p and -let7e-5p performing a ROC curve analysis, both as single predictors and in combination using a multivariable model to distinguish the DR-TB or MDR-TBs group from the group of samples from healthy individuals. We observed that miRNA-Model reached an Area Under the Curve value (AUC) = 0.96 (95% CI=0.907-1) for DR-TB patients (**Figure 3A** and **Table 2**) and AUC= 0.95, (95% CI= 0.89-1) for MDR-TB patients (**Figure 3B** and **Table 2**), showing to be a more sensitive discriminator between healthy and diseased individuals.

miRlet-7e-5p and miR328-3p Expression Change at the Time of *M. tuberculosis* Negative Culture in DR or MDR TB Patients

A sputum culture-negative conversion is currently the most objective indicator of response during early stage of TB treatment (22). Thus, miRNA expression at T(3-5) months was analyzed after all patients showed negative sputum cultures. At this time point, significant changes in miR-328-3p and -let-7e-5p levels were detected: miR-328-3p was upregulated, whereas miR-let-7e-5p was downregulated (**Figure 4A**). Since some expression levels for some patients did not change the same way, an additional analysis was performed in the subgroup of samples from MDR-TB patients, where a significant difference for miR-20a-3p, -328-3p and -let-7e-5p was observed (**Figure 4B**).

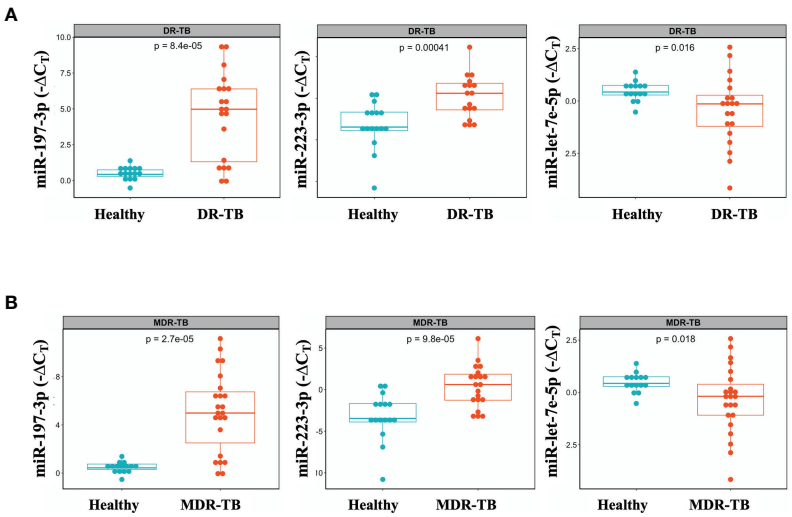


FIGURE 2 | Differential miR-let-7e-5p, miR-197-3p and miR-223-3p expression in serum exosomes from DR-TB patients and healthy subjects. Serum exosomes were isolated, miRNAs amplification was done by qPCR. The data were expressed as ΔC_T, defined as the relative expression of each miRNA with respect to the endogenous control (mirR-145-5p). Box plots depict medians and quartiles. **(A)** DR-TB patients (n = 24) and **(B)** MDR-TB patients (n = 20) and healthy subjects (n = 16), $p < 0.05$, (TB *vs.* Control), DR, Drug Resistant; MDR, Multi Drug Resistant. Raw C_T data were normalized, and expression differences between cases and controls were assessed by an unpaired two-samples Mann-Whitney test. Only statistical differences with $p < 0.05$ are shown.

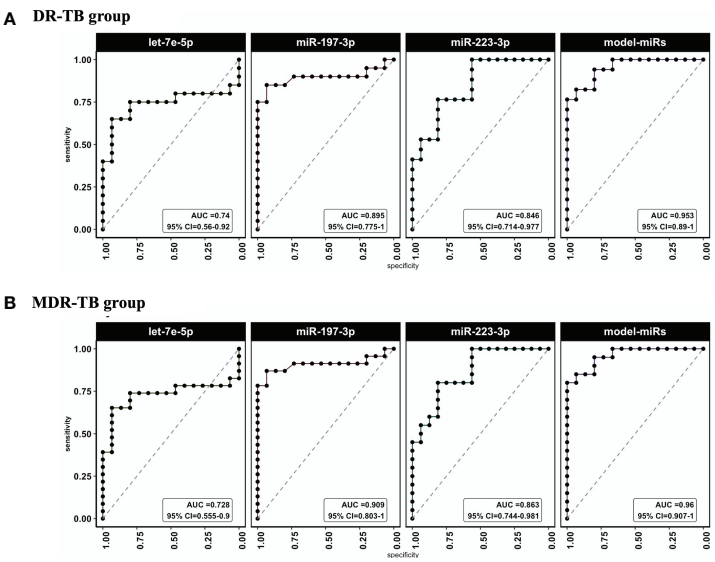


FIGURE 3 | ROC curves and AUC values for miRlet-7e-5p, -197-3p and -223-3p miRNAs in TB patients *vs* healthy subjects. ROC analysis was done to evaluate sensitivity and specificity. ROC curves were used to compare the relative expression of the three miRNAs in DR-TB and MDR-TB patients with those expressed in healthy subjects. **(A)** AUC and CI to miR-let-7e-5p, -197-3p and -223-3p for DR-TB patients and **(B)** (AUC) and CI to miR-let-7e-5p, -197-3p and -223-3p for MDR-TB patients. AUC, area under the curve; CI, confidence interval; ROC, receiver operator characteristic. Only statistical differences with $p < 0.05$ are shown.

Long-Term Treatment Monitoring Was Associated With miR-let7e-5p in the Group of MDR-TB Without T2DM

Treatment monitoring should be performed at least once a month during the intensive treatment phase (first six months), and then every 1 or 2 months during the continuation phase. Delay to identify treatment failure or the emergence of resistant strains of pulmonary TB leads to severe disease and transmission (22). No differences in the expression of miR-20a-3p, -195-5p, -197-3p, -223-3p, -328-3p, -451a and -let-7e-5p at T(6-8) or T(9-

TABLE 2 | ROC analysis for DR-TB or MDR-TB groups *vs* Healthy subjects.

Group	miR	AUC	AUCCI.min	AUCCI.max	Cutpoint (-ΔCt)	Sensitivity	Specificity
DR-TB	miR-197-3p	0.909	0.803	1	-0.054	0.652	0.933
	let-7e-5p	0.728	0.555	0.9	0.985	0.87	0.933
	223-3p	0.863	0.744	0.981	-1.523	0.8	0.812
	model-miRs(197-3p + let-7e-5p +223-3p)	0.96	0.907	1	0.714	0.8	1
MDR-TB	197-3p	0.895	0.775	1	-0.054	0.65	0.933
	let-7e-5p	0.74	0.56	0.92	0.985	0.85	0.933
	223-3p	0.846	0.714	0.977	-1.523	0.765	0.812
	model-miRs(197-3p + let-7e-5p +223-3p)	0.953	0.89	1	0.714	0.765	1

AUC, Area Under the Curve.

15) were found between the DR and MDR groups. Given that T2DM is a metabolic disorder that can affect biomarker assessment, we performed an additional analysis excluding patients with T2DM. As shown in **Figure 4C**, miR-let7e-5p was differentially expressed at early time points T(3-5), as well as at the late time points T(6-8) and T(9-15).

Transition Probabilities of miR-let-7e-5p Over Time Points as an Indicator of Changes

Since long-term identification of miRNAs could be an indicator of drug failure or relapse. an additional analysis was conducted to evaluate the transition pattern of miRNAs between timepoints. The diagram of comparisons or transitions between the timepoints (stages) is shown in **Figure 5A**. It was found that miR let-7e-5p is the only miRNA that reported an increase in δC_T values in most comparisons, maintaining a probability greater than 50%. The transitions of miR-let7e-5p to an upregulated or downregulated state over time are shown in a heatmap (**Figure 5B**). The probabilities for the miR let-7e-5p associated to the MDR-TB and MDR-TB-T2DM(-) groups are shown in **Figure 5C**. We found concordance between the changes observed in **Figure 5** with the transition probability analyses at the more distant T(6-8) and T(9-12) time points (**Figure 5**).

Presence of 30kDa and 5KST RNA From *M. tuberculosis* in Serum Exosomes of DR or MDR TB Patients

Although biomarkers based on the host response to *M. tuberculosis* are useful diagnostic tools, they may be affected by new or concomitant diseases. We found that the long-term expression of miR-let-7e-5p was associated only with MDR-TB patients without DM, and that some patients did not change in the same way. Therefore, it was necessary to search for a second pathogen-based marker.

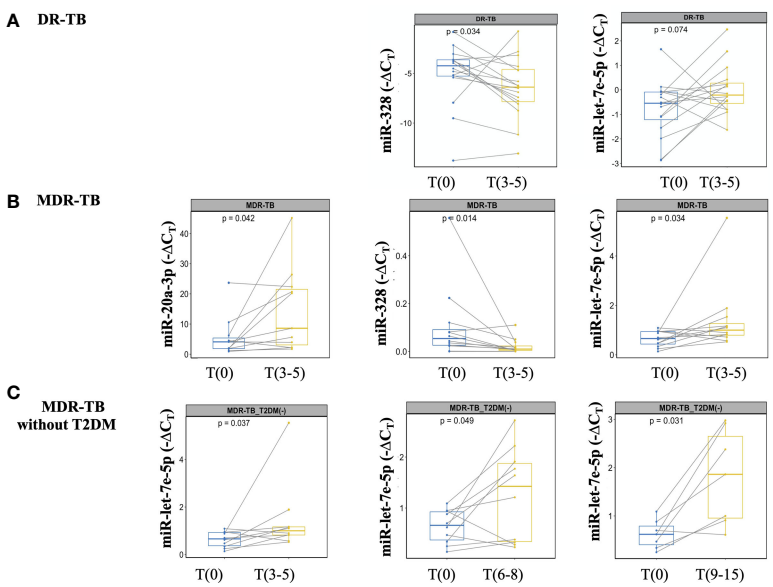
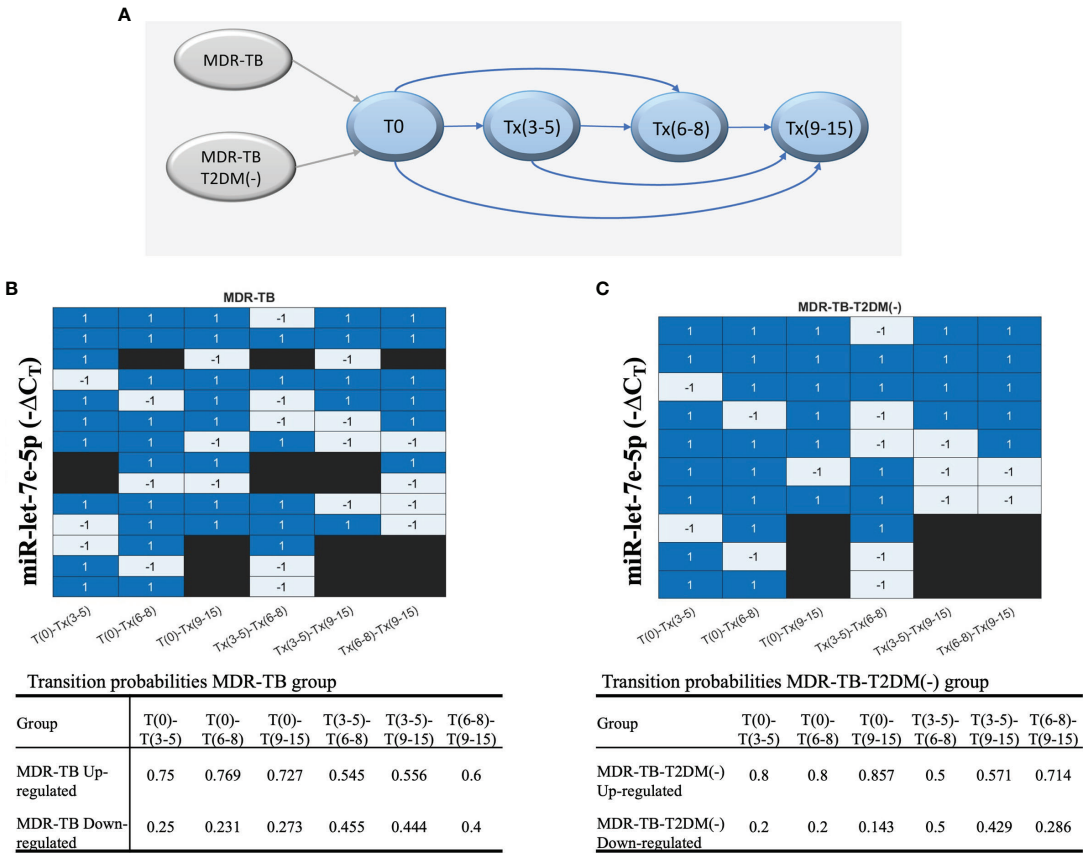


FIGURE 4 | miRNAs detection at the time of *M. tuberculosis* negative culture from DR-TB patients and treatment monitoring from MDR-TB without T2DM. miRNAs expression analyzed at T(0) and T(3-5). The data are expressed as ΔC_T, box plots depict medians and quartiles. Lines show the expression change for each patient. **(A)** DR-TB patients, **(B)** MDR-TB patients, and **(C)** MDR-TB without T2DM. Wilcoxon signed-rank test T(0) *vs.* T(3-5) or T(6-8) or T(9-15). Only statistical differences with $p < 0.05$ are shown.



Transition probabilities MDR-TB group

Group	T(0)-T(3-5)	T(0)-T(6-8)	T(0)-T(9-15)	T(3-5)-T(6-8)	T(3-5)-T(9-15)	T(6-8)-T(9-15)
MDR-TB Up-regulated	0.75	0.769	0.727	0.545	0.556	0.6
MDR-TB Down-regulated	0.25	0.231	0.273	0.455	0.444	0.4

Transition probabilities MDR-TB-T2DM(-) group

Group	T(0)-T(3-5)	T(0)-T(6-8)	T(0)-T(9-15)	T(3-5)-T(6-8)	T(3-5)-T(9-15)	T(6-8)-T(9-15)
MDR-TB-T2DM(-) Up-regulated	0.8	0.8	0.857	0.5	0.571	0.714
MDR-TB-T2DM(-) Down-regulated	0.2	0.2	0.143	0.5	0.429	0.286

FIGURE 5 | The behavior of each group of patients, showing the evolution of each patient according to the ΔC_T values during the periods of time. **(A)** The transitions model comparing six times. Upregulation or downregulation in the ΔC_T value between time points is illustrated in a heatmap. Blue: upregulation in the ΔC_T value in the next time point or the target time point; gray: downregulation in the ΔC_T Cs value, and black: no transition between time points. **(B)** MDR-TB group and **(C)** MDR-TB-T2DM (-) group. The table includes results of the transition probabilities of the two groups: MDR-TB, MDR-TB-T2DM(-). Each group is divided at the same time point into 2 subgroups according to the ΔC_T values of transition: upregulated or downregulated.

The presence of pathogen-derived RNA in exosomes released during macrophage infection has been reported (23). In view of the heterogeneity of the exosome cargo, we decided to evaluate the presence of proteins, DNA, and RNA sequences from TB patients. Neither DNA sequences (Mpb64, 5KST, 19kDa, CFP-10, ESAT-6, Mpt64, 30kDa and Ag85c) nor proteins (ESAT-6, CFP-10, 38kDa, Ag85b and MPT64) were found in serum exosomes from DR-TB patients (data not shown). Nevertheless, RNA sequences for 30kDa and 5KST antigens in the serum exosomes from DR-TB patients were found. **Figure 6** shows the time point of *M. tuberculosis* antigen expression and PCR products in a heat map (**Figure 6B**). Antigens were expressed before treatment in 50% of DR-TB patients. However, after the intensive phase of treatment, *M. tuberculosis* RNA sequences were detected in only 33%. These data could also suggest treatment failure or relapse.

A Dual Marker for Monitoring MDR-TB Treatment

Finally, miR-let-7e-5p expression and *M. tuberculosis* antigens detected in the exosomes of MDR-TB at T(9-15) were evaluated for concordance. **Figure 7A** shows the change in miR-let7e-5p expression over time from T(9-15) in MDR-TB patients; despite the significant differences, the expression of miR-let7e-5p did not increase in some patients. Thus, the data have been disaggregated into two subgroups: one for exosomes negative to *M. tuberculosis* (**Figure 7B**), in which an increase of miR-let7e-5p is observed in all patients, and one for exosomes positive to 30kDa or 5KST proteins, in which miR-let7e-5p expression decreased in 60% of the patients (**Figure 7C**).

DISCUSSION

Despite recent advances in TB research, diagnosis and treatment monitoring still remain a global challenge, further exacerbated by the emergence of multidrug-resistant (MDR-TB) and extensively drug-resistant (XDR-TB) strains (24). It has been reported that *M. tuberculosis* may use exosomes as a mechanism for releasing molecules into host cells. The molecules identified are proteins, peptides and small RNA sequences (miRNAs) derived from both

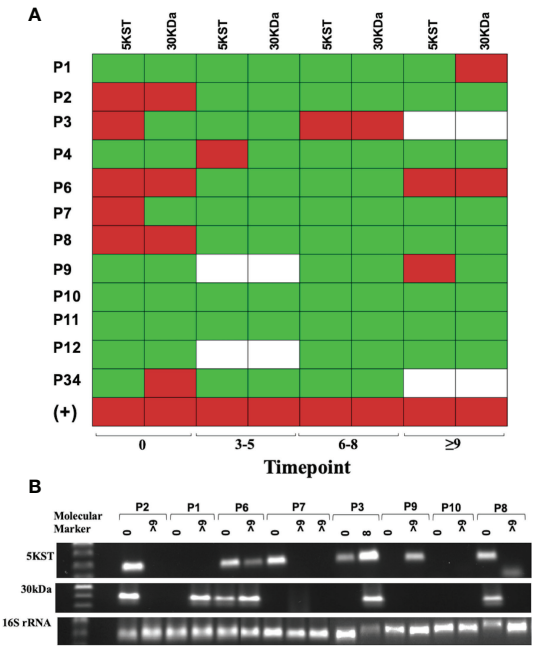


FIGURE 6 | Mycobacterial 5KST and 30kDa antigens are in exosomes from DR-TB patients. Representative results from amplification fragments are presented as a heat map for **(A)** 5KST and 30kDa (n =12). Red square = positive amplification; green square = negative amplification, and white square = non-determined. **(B)** Electrophoresis of 5KST, 30kDa and 16S rRNA amplification fragments.

host and pathogen (25). Host RNAs are present in exosomes during the *M. tuberculosis* infectious process, and are potential biomarkers for the detection or diagnosis of latent and active TB (26). We use these findings to propose a dual test to detect drug failure or the emergence of resistant strains during the monitoring of treatment.

The DR-TB diagnosis is based on microbiological testing in which the sputum specimina are smeared directly onto the slides and stained using the Ziehl-Neelsen (ZN) technique; in spite of a high specificity, the sensitivity of the test has been reported to vary from 20 to 80% (21). Culture of *M. tuberculosis* is the gold standard for diagnosis and cure of TB, as it is more sensitive than microscopy; however, around 10 bacteria/ml are needed, with a sensitivity of 80–85% and a specificity of approximately 98% (27, 28). The limitation of both methods is reflected on the relatively high proportion of patients who remain undiagnosed.

In this work, we propose the use of miRNAs from serum exosomes, which are non-invasive prognostic markers, and suggest the simultaneous evaluation of the three host derivate miRNAs (-let-7e-5p, -197-3p and -223-3p) for the identification of DR-TB (including RR, MDR and XDR) in those patients for whom the *M. tuberculosis* ZN staining or culture are negative. The expression of miR-197-3p and -223-3p are increased, while miR-let-7e-5p decreases in DR and MDR-TB patients. The expression of these three miRNAs is associated with an increased likelihood of MDR-TB in a multivariate model (ROC curve AUC= 0.96 and CI of 0.907-1). To our knowledge, this specific pattern of miRNAs expression had not been previously reported. It had been observed that miR-let-7e-5p, -let-7d-5p, -450a-5p, and -140-5p were expressed among patients with LTBI (28), while miR-223-3p was decreased in plasma samples from patients with TB compared to healthy controls (29). Now we are adding to those findings the observation that the expression of miR-let7e-3p is downregulated while miR-223-3p is increased in MDR-TB patients. According to our data and those published by Lingna et al., miR-let-7e-5p is suppressed during the active phase of the TB caused for any strain of *M. tuberculosis*, while its expression is increased in the TB-latent or after one year of drug treatment for MDR-TB patients, suggesting its use as a possible biomarker for non-resistant and resistant pulmonary TB (18).

miRNAs have regulatory functions: miR-223-3p targets the gene STAT1, involved in interferon-gamma signaling during TB disease (29); miR-let-7e-5p acts as a tumor suppressor, and its down-regulation may promote the development and progression of carcinomas (30). Finally, miR-197-3p binds to the interleukin-1beta (IL-1β) receptor, contributing to autoinflammatory processes (31). Nevertheless, the role of these miRNAs in tuberculosis remains to be determined.

To date, only microbiological methods based on sputum sample are available to monitor TB treatment; however, an important limitation of these methods lies in the availability of biological samples, since patients stop expectorating a few months after starting treatment. Furthermore, even if bacilli are detected again in sputum, few changes in the therapeutic regimen can be made (22). For this reason, we propose measuring miRNAs as part of the monitoring of DR-TB treatment: if at the time of sputum conversion T(3-5), the miR-let-7e-5p expression becomes downregulated, while the miR-328-3p is up-expressed, it is likely that RR, MDR or XDR strains have appeared. Additionally, we have shown that the up-expression of a particular miRNA, the miR-20a-3p, is associated with a specific type of resistance: that of the MDR group. Thus, miR-328-3p and -let-7e-5p expression could be useful to analyze in conjunction with other clinical and microbiological data to monitor DR-TB patients during the early stages of treatment.

To summarize, to evaluate early-term treatment efficacy, we propose to determine two miRNAs (miR-let-7e-5p and -328-3p) for all DR-TB patients and three miRNAs (miR-let-7e-5p, -328-3p and -20a-3p) for MDR-TB patients. The strategy of using a set of biomarkers had been suggested previously for MDR-TB diagnosis: specifically, with a model based on five biomarkers (CD44, KNG1, and miR-4433b-5p, -424-5p, and -199b-5p) (32). However, the latter study has important differences with our own, namely, the type of sample (serum) and the tools used (liquid chromatography-tandem mass spectrometry and sequencing).

The main challenge of this study was to find a biomarker to predict treatment failure or relapse after the completion of the intensive phase (22). Although we expected to find biomarkers that could be useful for all patients regardless of their comorbidities, we did not find any miRNA expression pattern applicable to the MDR-TB group in general, but only in MDR-TB patients without T2DM.

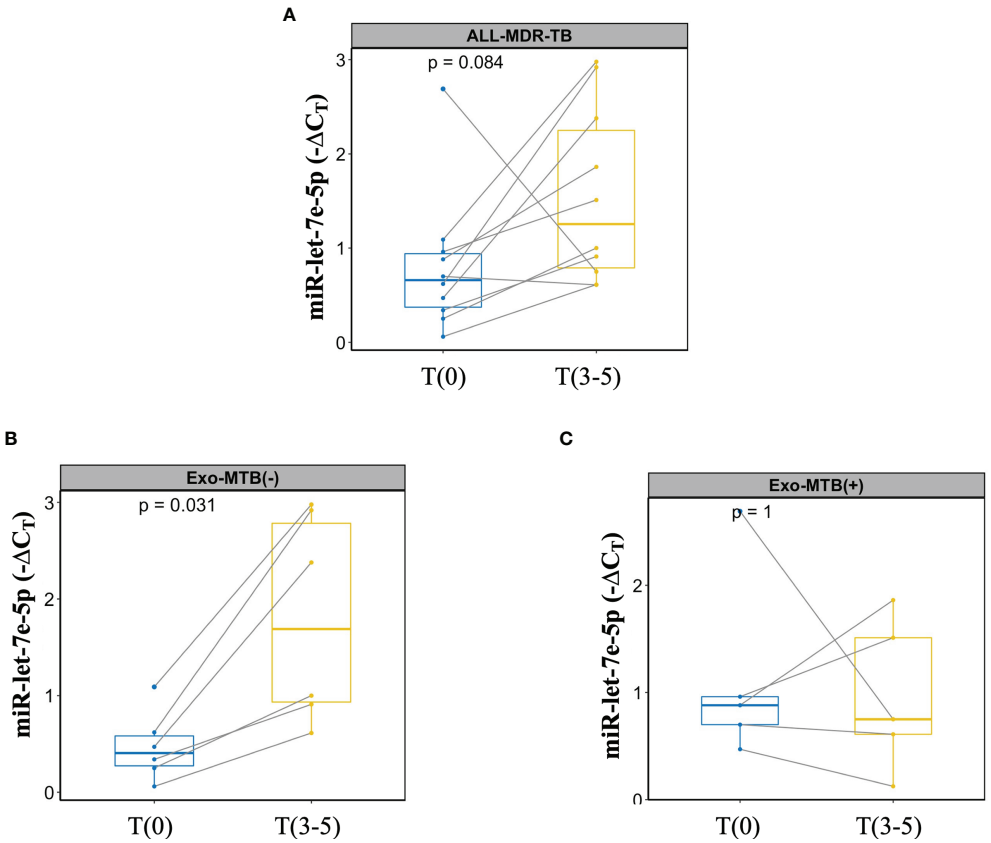


FIGURE 7 | Dual marker evaluation in patients with MDR-TB. Lines show the change in expression for each patient. **(A)** miR-let7e-5p expression in MDR-TB group, **(B)** MDR-TB patients negative for 30kDa or 5KST *M. tuberculosis* sequences. **(C)** MDR-TB patients positive for 30kDa or 5KST *M. tuberculosis* sequences.

In this group, we observed that miR-328-3p and -let7e-5p correlate with the time of smear conversion T(3-5), and that miR-let7e-5p correlates with the intensive phase of treatment T(9-15). Based on these data, miR-let7e-5p expression may be a biomarker in patients with MDR-TB without T2DM.

The presence of T2DM in TB patients not only tends to make biomarker assessment more difficult. On one hand, oral antihyperglycemic drugs can alter the pharmacokinetics, safety, and clinical effects of anti-TB drugs. On the other, T2DM patients are also at a higher risk of TB treatment failure, death, and relapse after cure. Therefore, treatment for TB-T2DM patients needs to be personalized to lower resistance rates and to have better outcomes (33, 34).

Additionally, the probabilities of transitions, comparing all the times studied, led to identify the miR let-7e-5p in both the MDR-TB and MDR-TB-T2DM(-) groups, with higher transition probability at the more distant T(6-8) and T(9-12) time points, thus supporting its use as a potential biomarker.

Despite the advantages of biomarkers as non-invasive diagnostic tools, circulatory miRNAs have been reported to be redundant to similar miRNAs in various diseases and to be influenced by external factors such as smoking, diet, circadian cycles, etc. This makes difficult to link circulatory miRNAs to specific diseases (35). For these reasons, we evaluated a second *M. tuberculosis*-derived marker of the presence of proteins, DNA and RNA sequences in the serum exosomes from MDR-TB patients. Thus, sequences were amplified for 30kDa and 5KST antigens of *M. tuberculosis* from exosomal-RNA at T(9-15) after the intensive phase of treatment. A concordance between the expression of miR-let-7e-5p and the *M. tuberculosis* antigens was observed in the exosomes of 60% of patients after the intensive phase of treatment. When the expression of miR-let7e-5p decreases, the RNA for either 30kDa or 5KST proteins are detected.

A limitation of this study lies on the heterogeneity of the patients, the reduced number of DR-TB patients, and the failure to detect a miRNA associated with T2DM. Nevertheless, the results may be useful in the non-diabetic population.

CONCLUSION

A dual marker presents in serum exosomes, consisting of host-derived miR-let7e-5p and *M. tuberculosis*-derived RNA for 30kDa or 5KST, may detect treatment failure or relapse for MDR-TB patients. We propose this double evaluation as a method of a long-term monitoring of TB treatment. Given the lack of methods

available for detection of drug failure or relapse, these tests could help clinicians to make adjustments to therapeutic regimens and improve the control of TB in their patients.

DATA AVAILABILITY STATEMENT

The original contributions presented in the study are included in the article/**Supplementary Material**. Further inquiries can be directed to the corresponding author.

ETHICS STATEMENT

The Ethics Committee from the Instituto Nacional de Enfermedades Respiratorias Ismael Cosío Villegas approved this study (No. C57-17). The patients/participants provided their written informed consent to participate in this study.

AUTHOR CONTRIBUTIONS

CC, MTH, MS-C, and YG made sample assays and data analysis. IS-G, ES, and SG-B analyzed and interpreted the data. LC-G analyzed the results. LHG-G and YG designed the study and wrote the manuscript. All authors contributed to the article and approved the submitted version.

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SUPPLEMENTARY MATERIAL

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Combination of HLA-DR on *Mycobacterium tuberculosis*-Specific Cells and Tuberculosis Antigen/Phytohemagglutinin Ratio for Discriminating Active Tuberculosis From Latent Tuberculosis Infection

Ying Luo[1], Ying Xue[2], Guoxing Tang[1], Qun Lin[1], Huijuan Song[1], Wei Liu[1], Botao Yin[1], Jin Huang[1], Wei Wei[1], Liyan Mao[1]*, Feng Wang[1]* and Ziyong Sun[1]**

[1] Department of Laboratory Medicine, Tongji Hospital, Tongji Medical College, Huazhong University of Science and Technology, Wuhan, China, [2] Department of Immunology, School of Basic Medicine, Tongji Medical College, Huazhong University of Science and Technology, Wuhan, China

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Edited by:
Novel N. Chegou,
Stellenbosch University, South Africa

Reviewed by:
Alasdair Leslie,
Africa Health Research Institute (AHRI), South Africa
Yonas Bekele,
National Institutes of Health (NIH), United States

***Correspondence:**
Liyan Mao
862200862@qq.com
Feng Wang
fengwang@tjh.tjmu.edu.cn
Ziyong Sun
zysun@tjh.tjmu.edu.cn
Ying Luo
13349917282@163.com

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Background: Novel approaches for tuberculosis (TB) diagnosis, especially for distinguishing active TB (ATB) from latent TB infection (LTBI), are urgently warranted. The present study aims to determine whether the combination of HLA-DR on *Mycobacterium tuberculosis* (MTB)-specific cells and TB antigen/phytohemagglutinin (TBAg/PHA) ratio could facilitate MTB infection status discrimination.

Methods: Between June 2020 and June 2021, participants with ATB and LTBI were recruited from Tongji Hospital (Qiaokou cohort) and Sino-French New City Hospital (Caidian cohort), respectively. The detection of HLA-DR on MTB-specific cells upon TB antigen stimulation and T-SPOT assay were simultaneously performed on all subjects.

Results: A total of 116 (54 ATB and 62 LTBI) and another 84 (43 ATB and 41 LTBI) cases were respectively enrolled from Qiaokou cohort and Caidian cohort. Both HLA-DR on IFN-γ^+TNF-α^+ cells and TBAg/PHA ratio showed discriminatory value in distinguishing between ATB and LTBI. Receiver operator characteristic (ROC) curve analysis showed that HLA-DR on IFN-γ^+TNF-α^+ cells produced an area under the ROC curve (AUC) of 0.886. Besides, TBAg/PHA ratio yield an AUC of 0.736. Furthermore, the combination of these two indicators resulted in the accurate discrimination with an AUC of 0.937. When the threshold was set as 0.36, the diagnostic model could differentiate ATB from LTBI with a sensitivity of 92.00% and a specificity of 81.82%. The performance obtained in Qiaokou cohort was further validated in Caidian cohort.

Conclusions: The combination of HLA-DR on MTB-specific cells and TBAg/PHA ratio could serve as a robust tool to determine TB disease states.

Keywords: *Mycobacterium tuberculosis*-specific cells, HLA-DR, TBAg/PHA ratio, discrimination, active tuberculosis, latent tuberculosis infection

INTRODUCTION

Tuberculosis (TB), caused by *Mycobacterium tuberculosis* (MTB) infection, remains an ongoing and leading global public issue with high morbidity and mortality (1). It was reported that the disease caused an estimated 10.0 million incident cases and approximately 1.4 million deaths worldwide in 2019 (2). Most subjects infected with MTB remain relatively healthy, a state called latent TB infection (LTBI) (3). However, approximately 5-10% of these individuals will eventually develop to active TB (ATB) during their life (4). Rapid approaches that can differentiate ATB from LTBI are essential for TB management and control, as well as the implement of the end TB strategy (5). Thus, developing effective and feasible methods become a current priority in combating the disease.

Currently, diagnosing ATB is mainly based on identifying the pathogen by staining for acid-fast bacilli, mycobacterial culture or PCR such as GeneXpert MTB/RIF. However, these methods are either insensitive or time-consuming, failing to meet clinical needs (6). Meanwhile, two kinds of commercial interferon gamma release assays, including QuantiFERON-TB Gold In-Tube test (QFT-GIT) and T-SPOT.TB (T-SPOT), were widely used for identifying MTB infection (7). Nevertheless, both T-SPOT and QFT-GIT are intrinsically unable to discriminate between ATB and LTBI well (8, 9). To address these limitations, emerging techniques, including transcriptomics (10, 11), proteomics (12, 13), and metabolomics (14, 15) have recently been introduced. However, these technique-derived tests lack sufficient validation and are difficult to carry out in clinical practice due to cumbersome operating procedures and requirements for special equipment (16). Therefore, despite much effort to identify new diagnostic methods for TB, we still lack affordable and efficient tools, especially based on existing platform, targeting this issue.

Surface markers on immune cells and intracellular cytokines detected by flow cytometry had been applied in TB diagnostic field in recent years (17, 18). Among these efforts, the activation phenotype represented by HLA-DR on MTB-specific cells appeared to be particularly outstanding in identifying ATB (19). However, in a more recent study, Mpande et al. denoted that HLA-DR on MTB-specific cells could not differentiate ATB patients from LTBI individuals with recent MTB infection (20). Thus, HLA-DR on MTB-specific cells would show relatively moderate specificity in distinguishing ATB from LTBI. Hence, there is a considerable need to seek a method with high specificity to combine with HLA-DR to compensate for its loss in specificity. Wang and his colleagues previously developed an indicator-TB antigen/phytohemagglutinin (TBAg/PHA) ratio, which showed relatively high specificity and moderate sensitivity in discriminating ATB patients from LTBI individuals (21, 22). We wonder whether the combination of these two indicators could further improve the differentiation. Therefore, the present study aims to investigate the potential value of the combination of HLA-DR on MTB-specific cells and TBAg/PHA ratio in distinguishing between ATB and LTBI.

METHODS

Subjects

The current study was conducted between June 2020 and June 2021. Participants were recruited from Tongji Hospital (Qiaokou cohort, the largest tertiary hospital in central China with 5500 beds) and Sino-French New City Hospital (Caidian cohort, a branch hospital of Tongji Hospital with 1600 beds), respectively. Participants in two cohorts were enrolled based on positive T-SPOT results. Patients with suspected symptoms of ATB and eventually confirmed by microbiological evidences were included. The definition for ATB was positive culture for MTB and/or positive GeneXpert MTB/RIF, as well as supportive symptoms and radiological evidence for ATB. LTBI individuals included in the current study were recruited from the populations who underwent health screening at hospitals. LTBI individuals were defined by positive T-SPOT results with no clinical or radiographic evidence of ATB (3, 23–25). Subjects were excluded if they had received anti-TB chemotherapy or younger than 17 years old. All enrolled subjects were HIV-negative. In order to determine the change of various indicators during anti-TB treatment, three months of anti-TB treatment was performed on ATB patients with isoniazid, rifampicin, pyrazinamide, and ethambutol. Three consecutive negative GeneXpert MTB/RIF results and relief of the patient's symptoms were considered signs of effective treatment. The study protocol was approved by the Ethics Committee of Tongji Hospital, Tongji Medical College, Huazhong University of Science and Technology. All participants provided written informed consent.

T-SPOT Assay

T-SPOT assay was performed using heparin-anticoagulated blood samples. The operation was conducted in accordance with manufacturer's instruction (Oxford Immunotec, Oxford, UK). Briefly, peripheral blood mononuclear cells (PBMCs) were separated by Ficoll-Hypaque gradient centrifugation. Then, the isolated PBMCs (2.5×10^5) were added to 96-well plates precoated with antibody against IFN-γ. There were four wells each participant: medium well (negative control), early secreted antigenic target 6 (ESAT-6) well (panel A), culture filtrate protein 10 (CFP-10) well (panel B), and PHA well (positive control). Cells were incubated for 16-20 h at 37°C with 5% CO_2

and developed using anti-IFN-γ antibody conjugate with substrate to detect the presence of IFN-γ secreted cells. Spot-forming cells (SFCs) were counted with an automated enzyme-linked immunospot reader (CTL Analyzers, Cleveland, OH, USA). The criteria for T-SPOT results were recommended by the manufacturer (26). The test result was positive if ESAT-6 and/or CFP-10 spot number minus negative control spot number ≥ 6. The test result was negative if both ESAT-6 spot number minus negative control spot number and CFP-10 spot number minus negative control spot number ≤ 5. Results were considered undetermined if the spot number in PHA well were < 20 or if spot number in the medium well were > 10. We calculated the ratios of (a) ESAT-6 SFCs to PHA SFCs and (b) CFP-10 SFCs to PHA SFCs. The larger of the above two values was defined as TBAg/PHA ratio of one subject.

Detection of HLA-DR on MTB-Specific Cells

PBMCs were stimulated with peptide ESAT-6 (2μg/ml) and CFP-10 (2μg/ml) for 18 hours at 37°C with 5% CO_2. Post incubation, PBMCs were first stained with Fixable Viability Stain 700 (BD Pharmingen) to differentiate live cells from dead cells, followed by appropriate surface marker staining. Cell surface staining was performed on PBMCs using the following anti-human monoclonal antibodies: anti-CD4-APC-Cy7 (Biolegend, Clone: RPA-T4; Cat# 300518) and anti-HLA-DR-PerCp-Cy 5.5 (Biolegend, Clone: L243; Cat# 307630). For intracellular staining, the cells were fixed and permeabilized with Fixation and Permeabilization Buffer (BD Biosciences). Intracellular cytokine staining was conducted using the flowing anti-human monoclonal antibodies: anti-IFN-γ-BV605 (Biolegend, Clone: 4S.B3; Cat# 502536) and anti-TNF-α-FITC (Biolegend, Clone: MAb11; Cat# 502906). Isotype controls with irrelevant specificities were included as negative controls. After washing, the pellets were resuspended in 300μl staining buffer and analyzed with FACSCanto II flow cytometer (BD Biosciences, San Jose, CA). The flow data were analyzed using Flowjo software version 10.6.2 (TreeStar, Ashland, OR). The gating strategy was showed in **Figure 1**.

MTB-specific cells were determined by IFN-γ and TNF-α co-producing cells upon TB antigen stimulation. Responders were regarded as cases with at least ten IFN-γ^+TNF-α^+ recorded events, and the proportion of IFN-γ^+TNF-α^+ cells after TB antigen stimulation was greater than 0.03% and was at least three times the frequency of that in the unstimulating control. HLA-DR expression analysis for MTB-specific cells were only performed on responders.

Statistical Analysis

Continuous variables were showed by median (interquartile range) or means ± standards deviation (SD). Categorical variables were expressed as number (%). Comparison between various groups was performed using Mann-Whitney *U* test for continuous variables, and Chi-square test or Fisher's exact test for categorical variables. Wilcoxon test was used to compare various indicators of the same patient before and after anti-TB treatment. *P* values of less than 0.05 were considered significant. To establish the diagnostic model for distinguishing ATB from LTBI, variables with statistical difference were taken as candidates for further multivariable logistic regression. Subsequently, the regression equation and diagnostic model were obtained and a predictive value for each individual was calculated. Receiver operator characteristic (ROC) curve analysis was performed to define the diagnostic performance of various biomarkers. Area under the ROC curve (AUC), sensitivity, specificity, positive predictive value (PPV), negative predictive value (NPV), positive likelihood ratio (PLR), negative likelihood ratio (NLR), and accuracy, together with their 95% confidence intervals (CI), were determined. The comparison between various ROC AUCs was performed using by z test with the procedure of Delong et al. (27). All statistical analysis were conducted using GraphPad Prism software version 8.0 (GraphPad, San Diego, CA), MedCalc version 11.6 (MedCalc, Mariakerke, Belgium), and SPSS software version 25.0 (SPSS, Chicago, IL).

RESULTS

Participants

A total of 116 subjects, including 54 ATB patients and 62 LTBI individuals were recruited at Qiaokou cohort. Another 84 cases, including 43 ATB patients and 41 LTBI individuals were enrolled in Caidian cohort. Demographic and clinical features of recruited participants in this study were shown in **Table 1**. The median age of included participants was around 50 years and more than half of these cases were males. No significant difference was observed between ATB and LTBI groups in distribution of age and gender among two cohorts.

HLA-DR on MTB-Specific Cells for Distinguishing ATB From LTBI

We compared HLA-DR expression on MTB-specific cells represented by IFN-γ^+TNF-α^+ cells upon TB antigen stimulation. It was observed that HLA-DR on IFN-γ^+TNF-α^+ cells was significantly higher in ATB group than that in LTBI group (**Figure 2A**). ROC curve analysis showed that HLA-DR on IFN-γ^+TNF-α^+ cells provided an AUC of 0.886 (95% CI, 0.826-0.946), with a sensitivity of 90.00% (95% CI, 78.64%-95.65%) and a specificity of 61.82% (95% CI, 48.61%-73.48%) to distinguish ATB from LTBI when a cut-off value of 49% was set (**Table 2** and **Figure 2B**). We stratified the subjects according to the age and found that there was no significant difference in HLA-DR expression on MTB-specific cells between individuals with young age (≤ 50 years old) and those with old age (> 50 years old) in both ATB and LTBI group (**Supplementary Figure 1A**).

TBAg/PHA Ratio for Differentiating ATB From LTBI

The value of TBAg/PHA ratio for differentiating ATB from LTBI was also assessed. ATB patients had significantly higher TBAg/PHA ratio compared to LTBI individuals (**Figure 2C**). ROC

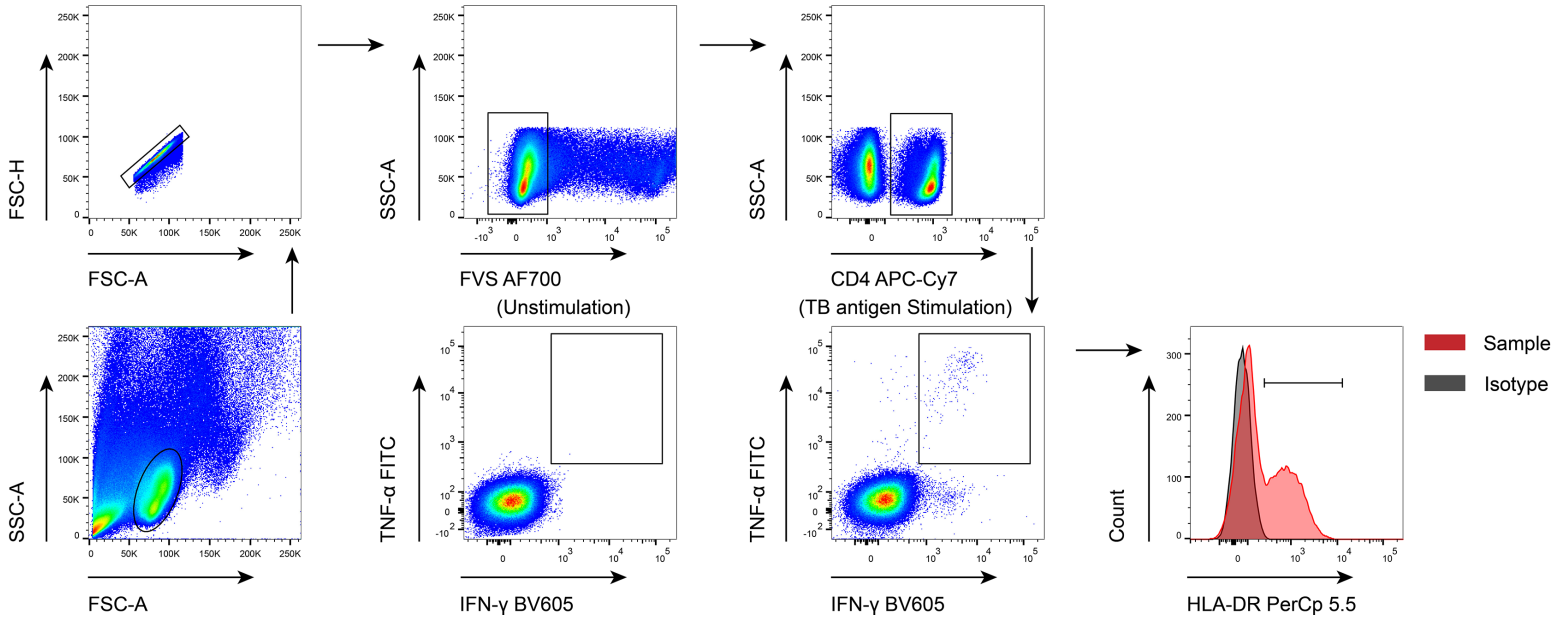

FIGURE 1 | The gating strategies used in the current study. FVS, flexible viability stain; TB, tuberculosis.

TABLE 1 | Demographic and clinical characteristics of recruited participants.

Variables	Qiaokou cohort (training set)		P*	Caidian cohort (validation set)		P*	P†
	ATB (n=54)	LTBI (n=62)		ATB (n=43)	LTBI (n=41)		
Age, years	55 (35-65)	53 (32-66)	0.835	49 (29-57)	49 (37-60)	0.45	0.102
Sex, male, %	35 (64.81%)	36 (58.06%)	0.457	30 (69.77%)	28 (68.29%)	0.884	0.253
Underlying condition or illness							
Diabetes mellitus	14 (25.93%)	9 (14.52%)	0.124	9 (20.93%)	5 (12.2%)	0.283	0.57
Solid tumor	3 (5.56%)	3 (4.84%)	0.805	3 (6.98%)	2 (4.88%)	0.956	0.94
Hematological malignancy	1 (1.85%)	0 (0%)	0.466	1 (2.33%)	0 (0%)	1	1
Virus hepatitis or cirrhosis	6 (11.11%)	3 (4.84%)	0.362	7 (16.28%)	4 (9.76%)	0.376	0.214
Heart disease	5 (9.26%)	3 (4.84%)	0.569	3 (6.98%)	2 (4.88%)	0.956	0.789
End-stage renal disease	4 (7.41%)	1 (1.61%)	0.283	2 (4.65%)	1 (2.44%)	0.966	0.918
Organ transplantation	2 (3.7%)	0 (0%)	0.215	1 (2.33%)	0 (0%)	1	1
Immunosuppressive condition‡	6 (11.11%)	4 (6.45%)	0.575	5 (11.63%)	4 (9.76%)	0.94	0.618
Positive culture for MTB	48 (88.89%)	N/A	N/A	40 (93.02%)	N/A	N/A	N/A
Positive GeneXpert MTB/RIF	42 (77.78%)	N/A	N/A	31 (72.09%)	N/A	N/A	N/A

*ATB, active tuberculosis; LTBI, latent tuberculosis infection; MTB, Mycobacterium tuberculosis; N/A, not applicable. *Comparisons were performed between ATB and LTBI groups using Mann-Whitney U test, Chi-square test, or Fisher's exact test. †Comparisons were performed between Qiaokou and Caidian cohorts using Mann-Whitney U test, Chi-square test, or Fisher's exact test. ‡Patients who underwent chemotherapy or took immunosuppressants within 3 months. Data were presented as medians (25th-75th) or numbers (percentages).*

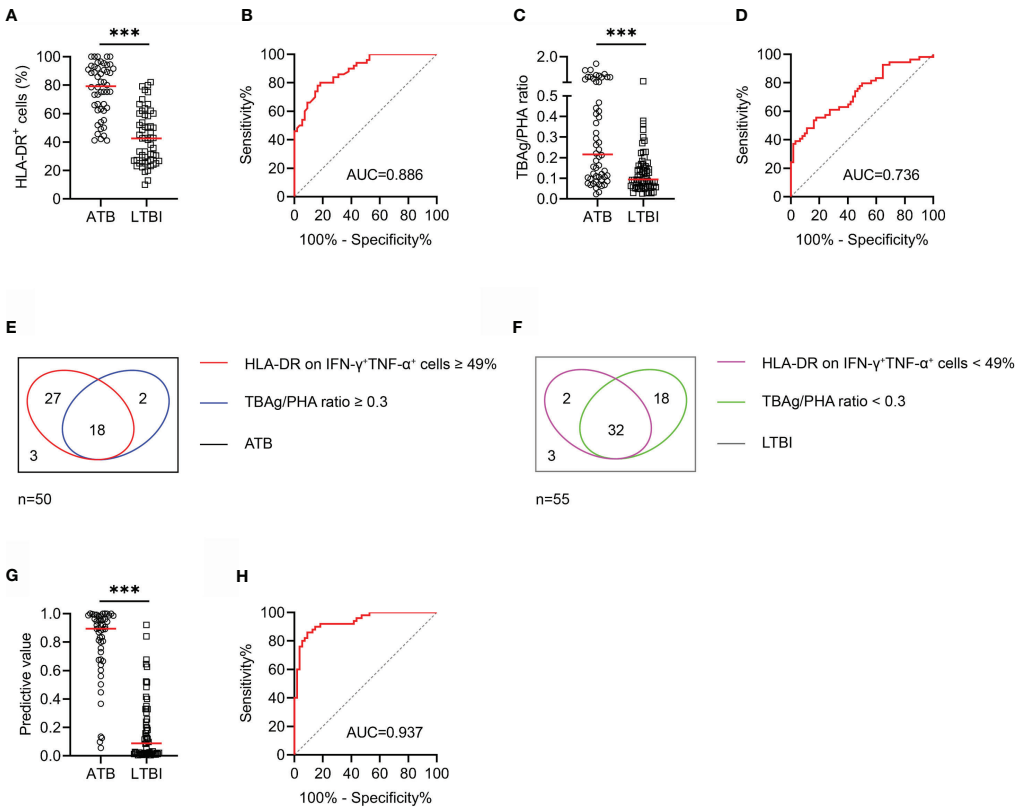

FIGURE 2 | The performance of various indicators in distinguishing ATB patients from LTBI individuals in Qiaokou cohort. **(A)** Scatter dot plots showing the results of the expression of HLA-DR on IFN-γ^+TNF-α^+ cells in ATB patients and LTBI individuals. Horizontal lines indicate the medians. ***$P < 0.001$ (Mann-Whitney *U* test). **(B)** ROC curve analysis showing the performance of HLA-DR on IFN-γ^+TNF-α^+ cells in discriminating ATB patients from LTBI individuals. **(C)** Scatter dot plots showing the results of TBAg/PHA ratio in ATB patients and LTBI individuals. Horizontal lines indicate the medians. ***$P < 0.001$ (Mann-Whitney *U* test). **(D)** ROC curve analysis showing the performance of TBAg/PHA ratio in discriminating ATB patients from LTBI individuals. **(E)** Venn diagrams showing the overlap of HLA-DR on IFN-γ^+TNF-α^+ cells and TBAg/PHA ratio in ATB patients. **(F)** Venn diagrams showing the overlap of HLA-DR on IFN-γ^+TNF-α^+ cells and TBAg/PHA ratio in LTBI individuals. **(G)** Scatter plots showing the predictive value of diagnostic model in ATB patients and LTBI individuals. Horizontal lines indicate the medians. ***$P < 0.001$ (Mann-Whitney *U* test). **(H)** ROC curve analysis showing the performance of diagnostic model based on the combination of HLA-DR on MTB-specific cells and TBAg/PHA ratio in discriminating ATB patients from LTBI individuals. MTB, *Mycobacterium tuberculosis*; ATB, active tuberculosis; LTBI, latent tuberculosis infection; TBAg, tuberculosis antigens; PHA, phytohemagglutinin; AUC, area under the curve.

TABLE 2 | The performance of various indicators for discriminating ATB from LTBI in Qiaokou cohort.

Variables	Cutoff value	AUC (95% CI)	Sensitivity (95% CI)	Specificity (95% CI)	PPV (95% CI)	NPV (95% CI)	PLR (95% CI)	NLR (95% CI)	Accuracy
HLA-DR on IFN-γ^+TNF-α^+ cells (%)	49	0.886 (0.826-0.946)	90.00% (78.64%-95.65%)	61.82% (48.61%-73.48%)	68.18% (56.21%-78.15%)	87.18% (73.3%-94.4%)	2.36 (1.66-3.34)	0.16 (0.07-0.38)	75.24%
TBAg/PHA ratio	0.3	0.736 (0.645-0.827)	40.74% (28.68%-54.03%)	91.94% (82.47%-96.51%)	81.48% (63.30%-91.82%)	64.04% (53.69%-73.24%)	5.05 (2.05-12.42)	0.64 (0.51-0.81)	68.10%
Diagnostic model	0.36	0.937 (0.892-0.982)	92.00% (81.16%-96.85%)	81.82% (69.67%-89.81%)	82.14% (70.16%-90.00%)	91.84% (80.81%-96.78%)	5.06 (2.87-8.92)	0.1 (0.04-0.25)	86.67%

ATB, active tuberculosis; LTBI, latent tuberculosis infection; TBAg, tuberculosis-specific antigen; PHA, phytohaemagglutinin; AUC, area under the curve; PPV, positive predictive value; NPV, negative predictive value; PLR, positive likelihood ratio; NLR, negative likelihood ratio; CI, confidence interval.

curve analysis showed that TBAg/PHA ratio had an AUC of 0.736 (95% CI, 0.645-0.827) for discriminating patients with ATB from LTBI individuals (**Figure 2D**). When 0.3 was used as the threshold, the sensitivity and specificity of TBAg/PHA ratio for distinguishing ATB from LTBI was 40.74% (95% CI, 28.68%-54.03%) and 91.94% (95% CI, 82.47%-96.51%), respectively (**Table 2**). We stratified the subjects according to the age and found that the values of TBAg/PHA ratio in individuals with young age (≤ 50 years old) were slightly higher than those with old age (> 50 years old) in both ATB and LTBI group. However, there was no significant difference (**Supplementary Figure 1B**).

Establishing Diagnostic Model Based on Combining MTB-Specific Cell HLA-DR and TBAg/PHA Ratio for Discriminating ATB From LTBI

We found both MTB-specific cell HLA-DR and TBAg/PHA ratio showed moderate performance in discrimination between ATB and LTBI. However, the overlap between MTB-specific cell HLA-DR and TBAg/PHA ratio was observed using Venn diagram analysis, suggesting the combination of these two indicators might further improve the diagnostic value (**Figures 2E, F**). To determine whether the combination of these two indicators could improve the ability to discriminate ATB from LTBI, we generated a diagnostic model using logistic regression. A diagnostic model was developed as the following: $P = 1/[1 + e^{-(0.094 \times \text{MTB-specific cell HLA-DR} + 6.761 \times \text{TBAg/PHA ratio} - 7.328)}]$ P, predictive value; e, natural logarithm. The diagnostic model yielded promising discriminatory potential with an AUC of 0.937 (95% CI, 0.892-0.982) (**Figures 2G, H**). When the threshold was set as 0.36, the model performed excellently with a sensitivity of 92.00% (95% CI, 81.16%-96.85%) and specificity of 81.82% (95% CI, 69.67%-89.81%) (**Table 2**).

Value of Identified Biomarkers in Monitoring Anti-TB Treatment

To evaluate whether these identified biomarkers have the potential to be used for TB treatment monitoring, we compared the levels of MTB-specific cell HLA-DR, TBAg/PHA ratio as well as predictive value of diagnostic model before and after anti-TB treatment. It was observed that the level of MTB-specific cell HLA-DR, TBAg/PHA ratio, and predictive value were all significantly decreased after standard anti-TB treatment (**Figure 3**).

Independent Validation of the Diagnostic Model

In order to validate the performance of the diagnostic model, another independent cohort was included for evaluation. Similar performance was obtained with HLA-DR on MTB-specific cells in Caidian cohort. MTB-specific cell HLA-DR distinguished ATB patients from LTBI individuals with an AUC of 0.917 (95% CI, 0.856-0.977) and demonstrated a sensitivity and specificity of 91.89% (95% CI, 78.70%-97.21%) and 63.89% (95% CI, 47.58%-77.53%), respectively (**Table 3** and **Figures 4A, B**). Meanwhile, TBAg/PHA ratio yielded a sensitivity of 37.21% (95% CI, 24.38%-52.14%) and specificity of 87.80% (95% CI, 74.46%-94.68%) in distinguishing ATB

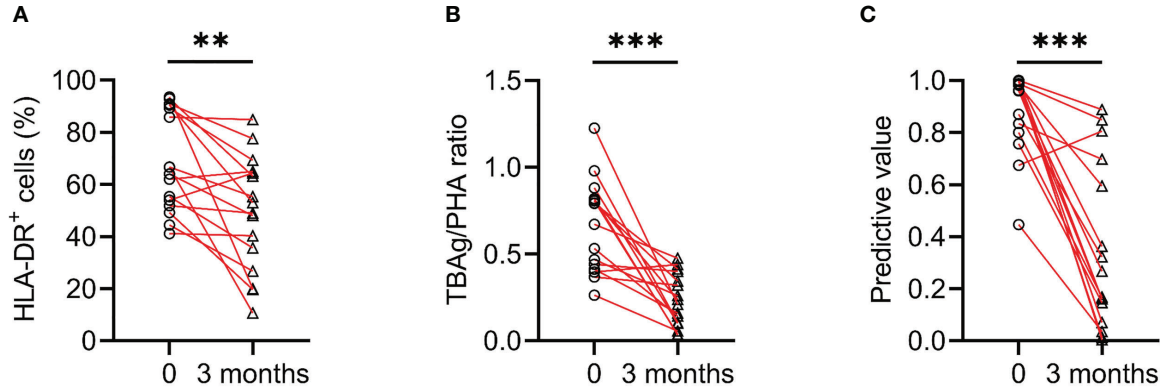

FIGURE 3 | The change of various indicators after anti-TB treatment. **(A)** Line graphs showing the expression of HLA-DR on IFN-γ^+TNF-α^+ cells in ATB patients before and after 3 months of anti-TB treatment. $^{**}P < 0.01$ (Wilcoxon test). **(B)** Line graphs showing the levels of TBAg/PHA ratio in ATB patients before and after 3 months of anti-TB treatment. $^{***}P < 0.001$ (Wilcoxon test). **(C)** Line graphs showing the predictive values of diagnostic model in ATB patients before and after 3 months of anti-TB treatment. $^{***}P < 0.001$ (Wilcoxon test). TBAg, tuberculosis antigens; PHA, phytohemagglutinin.

TABLE 3 | The performance of various indicators for discriminating ATB from LTBI in Caidian cohort.

Variables	Cutoff value	AUC (95% CI)	Sensitivity (95% CI)	Specificity (95% CI)	PPV (95% CI)	NPV (95% CI)	PLR (95% CI)	NLR (95% CI)	Accuracy
HLA-DR on IFN-γ^+TNF-α^+ cells (%)	49	0.917 (0.856-0.977)	91.89% (78.70%-97.21%)	63.89% (47.58%-77.53%)	72.34% (58.24%-83.06%)	88.46% (71.03%-96.00%)	2.54 (1.63-3.97)	0.13 (0.04-0.39)	78.08%
TBAg/PHA ratio	0.3	0.714 (0.605-0.824)	37.21% (24.38%-52.14%)	87.80% (74.46%-94.68%)	76.19% (54.91%-89.37%)	57.14% (44.86%-68.60%)	3.05 (1.23-7.57)	0.72 (0.55-0.92)	61.90%
Diagnostic model	0.36	0.941 (0.893-0.989)	91.89% (78.70%-97.21%)	86.11% (71.34%-93.92%)	87.18% (73.30%-94.40%)	91.18% (77.04%-96.95%)	6.62 (2.92-15.01)	0.09 (0.03-0.28)	89.04%

ATB, active tuberculosis; LTBI, latent tuberculosis infection; TBAg, tuberculosis-specific antigen; PHA, phytohaemagglutinin; AUC, area under the curve; PPV, positive predictive value; NPV, negative predictive value; PLR, positive likelihood ratio; NLR, negative likelihood ratio; CI, confidence interval.

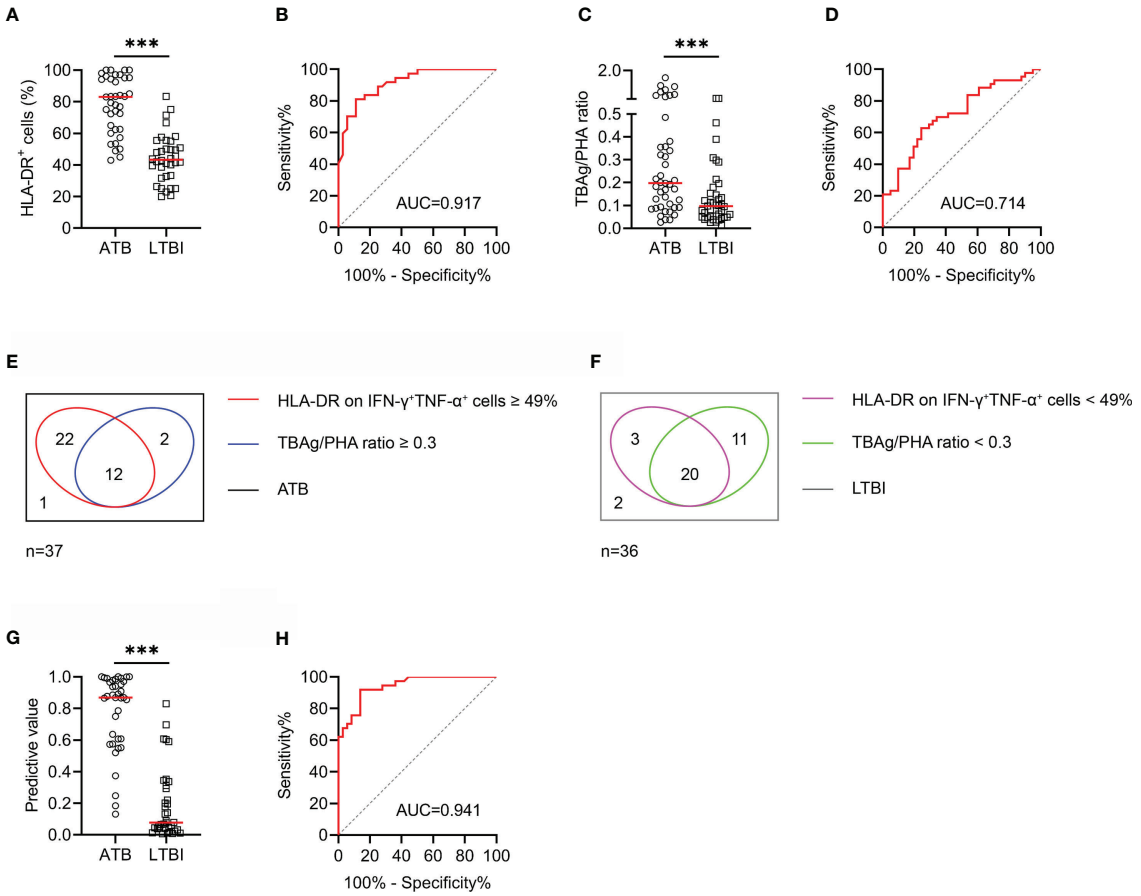

FIGURE 4 | The performance of various indicators in distinguishing ATB patients from LTBI individuals in Caidian cohort. **(A)** Scatter dot plots showing the results of the expression of HLA-DR on IFN-γ^+TNF-α^+ cells in ATB patients and LTBI individuals. Horizontal lines indicate the medians. ***$P < 0.001$ (Mann-Whitney *U* test). **(B)** ROC curve analysis showing the performance of HLA-DR on IFN-γ^+TNF-α^+ cells in discriminating ATB patients from LTBI individuals. **(C)** Scatter dot plots showing the results of TBAg/PHA ratio in ATB patients and LTBI individuals. Horizontal lines indicate the medians. ***$P < 0.001$ (Mann-Whitney *U* test). **(D)** ROC curve analysis showing the performance of TBAg/PHA ratio in discriminating ATB patients from LTBI individuals. **(E)** Venn diagrams showing the overlap of HLA-DR on IFN-γ^+TNF-α^+ cells and TBAg/PHA ratio in ATB patients. **(F)** Venn diagrams showing the overlap of HLA-DR on IFN-γ^+TNF-α^+ cells and TBAg/PHA ratio in LTBI individuals. **(G)** Scatter plots showing the predictive value of diagnostic model in ATB patients and LTBI individuals. Horizontal lines indicate the medians. ***$P < 0.001$ (Mann-Whitney *U* test). **(H)** ROC curve analysis showing the performance of diagnostic model based on the combination of HLA-DR on MTB-specific cells and TBAg/PHA ratio in discriminating ATB patients from LTBI individuals. MTB, *Mycobacterium tuberculosis*; ATB, active tuberculosis; LTBI, latent tuberculosis infection; TBAg, tuberculosis antigens; PHA, phytohemagglutinin; AUC, area under the curve.

patients from LTBI individuals (**Table 3** and **Figures 4C, D**). Furthermore, the diagnostic model produced an AUC of 0.941 (95% CI, 0.893-0.989), with a sensitivity of 91.89% (95% CI, 78.70%-97.21%) and a specificity of 86.11% (95% CI, 71.34%-93.92%) when a threshold of 0.36 was used (**Table 3** and **Figures 4E–H**).

We also analyzed the pooled diagnostic performance of various indicators when combining two cohorts. It was observed that the sensitivity and specificity of MTB-specific cell HLA-DR for discriminating ATB from LTBI were 90.80% (95% CI, 82.89%-95.27%) and 62.64% (95% CI, 52.38%-71.88%), respectively (**Supplementary Table 1** and **Supplementary Figures 2A, B**). TBAg/PHA ratio distinguished ATB from LTBI with a sensitivity of 39.18% (95% CI, 30.05%-49.12%) and a specificity of 90.29% (95% CI, 83.05%-94.64%) (**Supplementary Table 1** and **Supplementary Figures 2C, D**). Moreover, the diagnostic model produced an AUC of 0.940 (95% CI, 0.907-0.972) in differentiating ATB from LTBI, with a sensitivity of 91.95% (95% CI, 84.31%-96.05%) and a specificity of 83.52% (95% CI, 74.57%-89.75%) (**Supplementary Table 1** and **Supplementary Figures 2E, F**).

DISCUSSION

Despite decades of research and significant investment, there still exists a huge gap in meeting clinical need for diagnosing TB. The conventional tools to diagnose ATB have major limitations including inadequate utility, high cost as well as long turnaround time (28). Meanwhile, an increasing number of studies denoted that host signature, especially omics, could

serve as an alternative to pathogen detection (29). Identifying omics signatures for diagnosing TB have been facilitated by advances in technology to analyze obtained data using quantitative and high-throughput manner (30–33). However, even with numerous reports on novel candidates or multiple biomarker signatures, few of them have been independently validated for routine clinical use, and translated into applicable diagnostic tests. To achieve better management and control for TB, novel diagnostic strategies, especially based on present technology platform, are warranted.

Recently, MTB-specific cell signature such as activation and differentiation had been identified for the diagnosis of ATB (34–36). The available evidence indicated that HLA-DR on MTB-specific cells might be the most promising biomarker (37). Our data in this study also confirmed the discriminatory role of this indicator in distinguishing between ATB and LTBI. Notwithstanding, far less is known about the reliability and ability of combining MTB-specific cell HLA-DR and other indicators for the differential diagnosis between ATB and LTBI. To our knowledge, the present study established diagnostic model based on the combination of HLA-DR on MTB-specific cells and TBAg/PHA ratio for the first time. Our results demonstrated that the combination could further improve the diagnostic value. Moreover, we evaluated the value of these biomarkers in monitoring anti-TB treatment. The significant change with treatment denoted their potential value for monitoring therapy responses.

Two points should be pointed out in the present study. Firstly, we did find a high sensitivity and a moderate specificity of MTB-specific cell HLA-DR expression for differentiating ATB from LTBI. However, this seemed to be inconsistent with some previous studies, which showed that HLA-DR is a superior indicator with both excellent sensitivity and specificity (19). Nevertheless, we seemed to find an answer from the study conducted by Mpande and his/her colleagues (20). They reported that MTB-specific cells, from the LTBI population who were infected with MTB recently, also showed high activation, suggesting that the loss of specificity exhibited by HLA-DR in distinguishing ATB from LTBI is due to the presence of this population. Yet these cases did not show high values of TBAg/PHA ratio. Therefore, the combination of these two indicators could mainly contribute to improving the specificity, not the sensitivity. Second, we found that the sensitivity of TBAg/PHA ratio on distinguishing ATB from LTBI in this study was obviously lower than those reported in several previous studies (22), while similar with the utility obtained in a recent real-world data analysis from China (38) and another study from Japan (39). It was observed that the studies with good performance were often performed with patient exclusion such as immunosuppression. Therefore, the value of TBAg/PHA ratio in clinical application is mainly reflected in its acceptable specificity. In other words, TBAg/PHA ratio should be used more as a rule-in tool for its high specificity, rather than a rule-out test.

An optimal biomarker-based test would ideally be feasible with limited instrumentation and based on easily accessible samples such as peripheral blood. The methods involved in our diagnostic model fit the requirement. However, several limitations should be mentioned in this study. First, although two centers were included in our design, the number of subjects per center was too small and further validation with a large sample size is still needed in the future. Second, our model may not be helpful for T-SPOT-negative TB patients due to the lack of sufficient detectable MTB-specific cells in this population (40). More suitable tools should be developed targeting this population. Finally, since the established model was built based on two different techniques, the economic and clinical usefulness remain to be clarified in the further research.

In conclusion, the diagnostic model based on the combination of HLA-DR on MTB-specific cells and TBAg/PHA ratio would represent a new era of prompt TB diagnosis as an excellent auxiliary tool and enable earlier treatment, and thus reduce the spread of the disease, contributing toward paving the way for ending TB epidemic.

DATA AVAILABILITY STATEMENT

The original contributions presented in the study are included in the article/**Supplementary Material**. Further inquiries can be directed to the corresponding authors.

ETHICS STATEMENT

The studies involving human participants were reviewed and approved by the Ethics Committee of Tongji Hospital, Tongji Medical College, Huazhong University of Science and Technology. The patients/participants provided their written informed consent to participate in this study.

AUTHOR CONTRIBUTIONS

YL: had the original idea of this study. YL: conceived and designed the study. YL, YX, BY, JH, and WW: performed the experiments. YL: performed data collection and analysis. YL: wrote the manuscript. YL, GT, QL, HS, WL, and LM: enrolled participants. YL, FW, and ZS: contributed to revisions of the manuscript. All authors contributed to the article and approved the submitted version.

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SUPPLEMENTARY MATERIAL

The Supplementary Material for this article can be found online at: https://www.frontiersin.org/articles/10.3389/fimmu.2021.761209/full#supplementary-material

Supplementary Figure 1 | The results of various indicators in different age range. **(A)** Scatter dot plots showing the results of the expression of HLA-DR on IFN-γ^+TNF-α^+ cells under young age (≤ 50 years old) and old age (> 50 years old) in ATB patients and LTBI individuals. Horizontal lines indicate the medians. **(B)** Scatter dot plots showing the results of TBAg/PHA ratio under young age (≤ 50 years old) and old age (> 50 years old) in ATB patients and LTBI individuals. Horizontal lines indicate the medians.

Supplementary Figure 2 | The pooled performance of various indicators in distinguishing ATB patients from LTBI individuals. **(A)** Scatter dot plots showing the results of the expression of HLA-DR on IFN-γ^+TNF-α^+ cells in ATB patients and LTBI individuals. Horizontal lines indicate the medians. ***$P < 0.001$ (Mann-Whitney *U* test). **(B)** ROC curve analysis showing the performance of HLA-DR on IFN-γ^+TNF-α^+ cells in discriminating ATB patients from LTBI individuals. **(C)** Scatter dot plots showing the results of TBAg/PHA ratio in ATB patients and LTBI individuals. Horizontal lines indicate the medians. ***$P < 0.001$ (Mann-Whitney *U* test). **(D)** ROC curve analysis showing the performance of TBAg/PHA ratio in discriminating ATB patients from LTBI individuals. **(E)** Scatter plots showing the predictive value of diagnostic model in ATB patients and LTBI individuals. Horizontal lines indicate the medians. ***$P < 0.001$ (Mann-Whitney *U* test). **(F)** ROC curve analysis showing the performance of diagnostic model based on the combination of HLA-DR on MTB-specific cells and TBAg/PHA ratio in discriminating ATB patients from LTBI individuals. MTB, *Mycobacterium tuberculosis*; ATB, active tuberculosis; LTBI, latent tuberculosis infection; TBAg, tuberculosis antigens; PHA, phytohemagglutinin; AUC, area under the curve.

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Candidate Biomarkers to Distinguish Spinal Tuberculosis From Mechanical Back Pain in a Tuberculosis Endemic Setting

Theresa N. Mann[1,2], Johan H. Davis[1,2], Gerhard Walzl[3], Caroline G. Beltran[3], Jacques du Toit[1], Robert P. Lamberts[1,4] and Novel N. Chegou[3]*

[1] Division of Orthopaedic Surgery, Department of Surgical Sciences, Faculty of Medicine and Health Sciences, Stellenbosch University, Cape Town, South Africa, [2] Institute of Orthopaedics and Rheumatology, Mediclinic Winelands Orthopaedic Hospital, Stellenbosch, South Africa, [3] DSI-NRF Centre of Excellence for Biomedical Tuberculosis Research, South African Medical Research Council Centre for Tuberculosis Research, Division of Molecular Biology and Human Genetics, Faculty of Medicine and Health Sciences, Stellenbosch University, Cape Town, South Africa, [4] Department of Sport Science, Faculty of Medicine and Health Sciences, Stellenbosch University, Stellenbosch, South Africa

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***Correspondence:**
Theresa N. Mann
tmann@sun.ac.za

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Background: Spinal tuberculosis (TB) may have a variable, non-specific presentation including back pain with- or without- constitutional symptoms. Further tools are needed to aid early diagnosis of this potentially severe form of TB and immunological biomarkers may show potential in this regard. The aim of this study was to investigate the utility of host serum biomarkers to distinguish spinal TB from mechanical back pain.

Methods: Patients with suspected spinal TB or suspected mechanical back pain were recruited from a tertiary hospital in the Western Cape, South Africa, and provided a blood sample for biomarker analysis. Diagnosis was subsequently confirmed using bacteriological testing, advanced imaging and/or clinical evaluation, as appropriate. The concentrations of 19 host biomarkers were evaluated in serum samples using the Luminex platform. Receiver Operating Characteristic (ROC) curves and General Discriminant Analysis were used to identify biomarkers with the potential to distinguish spinal TB from mechanical back pain.

Results: Twenty-six patients with spinal TB and 17 with mechanical back pain were recruited. Seven out of 19 biomarkers were significantly different between groups, of which Fibrinogen, CRP, IFN-γ and NCAM were the individual markers with the highest discrimination utility (Area Under Curve ROC plot 0.88-0.99). A five-marker biosignature (CRP, NCAM, Ferritin, CXCL8 and GDF-15) correctly classified all study participants after leave-one-out cross-validation.

Conclusion: This study identified host serum biomarkers with the potential to diagnose spinal TB, including a five-marker biosignature. These preliminary findings require validation in larger studies.

Keywords: tuberculosis, spine, biomarkers, back pain, inflammation, cytokines

INTRODUCTION

Tuberculosis (TB) remains a global health priority with an estimated 10 million people worldwide developing TB disease in 2019 (1). Spinal TB is the most common form of osteoarticular TB and involves a chronic inflammatory process that gradually destroys the bony tissue of one or more vertebrae (2). With progression of the disease, those affected may develop serious sequelae such as spinal deformity, spinal instability and neurological deficit (2). These individuals may require costly corrective surgery and are at risk of significant long-term morbidity (2). Conversely, individuals with less advanced disease may be treated with TB medication alone and show largely favorable outcomes (3). Although spinal TB represents only 1-3% of all TB cases (4–6), the absolute number of individuals affected may be considerable in countries with a high burden of TB (7–9), creating a need for vigilance and effective methods of screening and early diagnosis.

Spinal TB is an insidious disease with a variable and non-specific clinical presentation, factors that contribute to a typical diagnostic delay of 4-12 months or more (6–8, 10–12). The most common symptom is chronic back pain, with 83-100% of individuals presenting with this complaint (7, 8, 11–14). However, previous reports suggest that only approximately 23-56% of patients have constitutional symptoms (e.g. fever, weight loss) (7, 8, 11–14) and 7-23% concomitant pulmonary TB (8, 10, 13, 14). With progression of the spinal TB disease, some patients may develop clinical signs such as kyphotic deformity (7, 8, 11–14). However, for the purposes of early diagnosis, there may not always be obvious features to help distinguish spinal TB amidst a high prevalence of mechanical back pain.

Conventional tools to help screen for pathological back pain at primary care level include evaluation of "red flags" and, when available, blood tests for erythrocyte sedimentation rate (ESR) and/or C-reactive protein (CRP) (15, 16). Red flags are patient factors and clinical signs thought to increase the likelihood of serious disease and include items such as unexplained weight loss (17–19). However, the list of potential red flags is lengthy, may vary with different clinical guidelines and is seldom supported by evidence for the diagnostic accuracy of the items (19). For example, there is a paucity of evidence for the effectiveness of red flags in detecting spinal infections (18). Furthermore, this screening approach may have limited sensitivity with a previous study reporting that the absence of red flags did not decrease the probability of a serious spinal pathology diagnosis (20). When considering ESR and CRP, these markers are elevated in most patients with spinal TB but may remain normal in some cases (12). Furthermore, these are non-specific tests of inflammation and patients may present with elevated levels for a variety of other reasons (15, 16).

Given the limitations of existing screening tools, there could be a significant benefit in more accurate point-of-care triage tests to indicate a high probability of spinal TB or diagnostic tools to identify spinal TB, depending on the performance characteristics of such new tools. One approach that may have potential in this regard is to explore a wider variety of diagnostic blood-based biomarkers than ESR and CRP and, in particular, multi-biomarker signatures. This methodology has received considerable attention in the context of pulmonary TB with certain immune biomarkers showing strong diagnostic potential (21–25). For example, in a recent pan-African study, a two-biomarker signature had a sensitivity of 93% and a specificity of 68% for the diagnosis of pulmonary TB regardless of HIV status (26). The ultimate aim of the afore-mentioned research is to develop cost-effective point-of-care triage or diagnostic tests for pulmonary TB in resource-limited settings (22, 27). However, it is possible that a similar approach could be used to assist with other diagnoses such as spinal TB.

Whereas biomarkers for pulmonary TB have been widely reported (21–26), less is known about biomarker profiles in specific forms of extra-pulmonary TB (EPTB), including spinal TB. Furthermore, the few existing studies describing biomarkers in spinal TB did not explore the diagnostic potential of the biomarkers by comparing them between spinal TB and other back pain etiologies (28, 29). The main aim of the current study was to investigate the utility of host biomarkers to distinguish spinal TB from mechanical back pain. A secondary aim was to test the performance of the afore-mentioned two-biomarker signature for pulmonary TB diagnosis (26) when applied to identify spinal TB among the current participants. It was envisaged that the findings of this exploratory study would identify candidate biomarkers for further evaluation as tools for spinal TB screening and diagnosis.

MATERIALS AND METHODS

Study Design and Setting

This case-control study was conducted at Tygerberg Hospital, a major tertiary hospital in the Western Cape Province of South Africa, between August 2016 and December 2018. In 2019, South Africa had an estimated TB incidence of 615 cases per 100 000 population and was ranked among the top eight countries in world for overall TB burden (1). At least 393 patients with spinal TB were referred to tertiary hospitals in the Western Cape between 2012 and 2015 (9) and, in 2016-2017, spinal TB admissions accounted for 21% of all spine unit admissions to the Tygerberg Hospital (30).

Participants

Patients with suspected spinal TB were recruited from the orthopedic wards of the hospital according to the following inclusion criteria: ≥ 18 years old, suspected spinal TB based on clinical presentation and Magnetic Resonance Imaging (MRI) findings, and not yet on TB treatment. An exclusion criterion was a subsequent spinal pathology diagnosis other than spinal TB. Patients with suspected mechanical back pain were recruited from the hospital's spine outpatient clinic according to the inclusion criteria: ≥ 18 years old and ≥ 3 months of chronic back pain of unknown cause. Patients subsequently diagnosed with non-mechanical back pain were excluded.

Clinical and Demographic Information

Clinical and demographic information for each participant was obtained using a standard intake interview and review of medical records. HIV status and, if applicable, most recent CD4 T-Lymphocyte and HIV viral load levels were obtained from medical records and laboratory test results. Furthermore, the Oswestry Disability Index (31) was completed with each participant in the form of a structured interview in order to provide a measure of disability related to back pain.

Spine Pathology Diagnosis

Participants with suspected spinal TB underwent an in-theatre spine biopsy for laboratory testing, according to the hospital's standard procedure. Each participant was subsequently classified as having bacteriologically confirmed- or clinically diagnosed-spinal TB, according to the World Health Organization's TB reporting definitions (32). Bacteriological confirmation was based on a positive GeneXpert and/or TB culture result from the spine biopsy. Conversely, a clinical diagnosis was made when bacteriological tests were negative for TB but did not present an alternative diagnosis plus factors such as clinical presentation, MRI findings, spine biopsy histology and/or TB bacteriologically confirmed at another site were suggestive of spinal TB.

For participants with suspected mechanical back pain, the diagnosis was based on the evaluation of an orthopedic specialist, including elements such as clinical history, a physical examination, X-rays and, when clinically indicated, advanced imaging.

QuantiFERON TB Gold Testing

Each participant provided a blood sample for QuantiFERON TB Gold In Tube (QFT) analysis. Blood was collected by venipuncture into QFT tubes (1ml per tube), followed by incubation for 16-24 hours at 37°C in a 5% CO_2 environment as recommended by the manufacturer (Qiagen, Germany). Thereafter, the tubes were centrifuged at 3000 RCF for 15 min, and supernatants harvested and stored at -80°C until used. Interferon gamma (IFN-γ) responses in the QFT supernatants were measured using the QFT ELISA kit and analyzed and interpreted according to the manufacturer's software. QFT tests were not standard procedure in the hospital and were conducted for the purpose of the research study.

Multiplex Host Biomarker Analysis

Blood for investigation of host cytokine levels was collected in 6 ml vacutainer serum tubes (BD Biosciences) and transported to the laboratory at ambient temperatures within 2 hours of collection. Tubes were subsequently centrifuged at 2000 RCF for 10 min and the serum aliquoted into micro-tubes. Aliquots were then stored at -80°C until analysis.

Nineteen host biomarkers were included in the Luminex immunoassay, as listed in **Table 1**. For the most part, these biomarkers were identified from existing TB biomarker literature (22, 27, 33). However, OPG was included due to its role in bone remodeling (34). It was hypothesized that markers of bone metabolism may be of value in differentiating spinal TB given the pathogenesis of this type of TB.

Experiments were conducted in a blinded manner by a Luminex-certified technician, in an ISO15189 accredited laboratory, using the Bio-Plex platform (Bio-Rad Laboratories, Hercules, USA), and were performed according to the kit manufacturer's instructions. Bio-Plex Manager software version 6.1 was used for bead acquisition and for the analysis of median fluorescence intensities.

TABLE 1 | Host biomarkers included in the Luminex immunoassay.

Abbreviation	Full name	Catalogue number	Detection limit
Biomarkers in kits purchased from R&D Systems Inc., Minneapolis, MN, USA			
CCL1 (I-309)	Chemokine (C-C motif) ligand 1	LXSAHM-11	0.119 pg/ml
CXCL8 (IL-8)	Chemokine (C-X-C motif) ligand 8 (Interleukin 8)	LXSAHM-11	1.8 pg/ml
CXCL9 (MIG)	Chemokine (C-X-C motif) ligand 9 (monokine induced by gamma interferon)	LXSAHM-11	23.8 pg/ml
CXCL10 (IP10)	Chemokine (C-X-C motif) ligand 10 (Interferon gamma-induced protein 10)	LXSAHM-11	1.18 pg/ml
Factor D (Adipsin)	Factor D (Adipsin)	LXSAHM-03	232 pg/ml
Ferritin	Ferritin	LXSAHM-03	1.29 pg/ml
GDF-15	Growth Differentiation Factor 15	LXSAHM-11	1.2 pg/ml
ICAM-1	Intercellular Adhesion Molecule 1	LXSAHM-11	87.9 pg/ml
IFN-γ	Interferon gamma	LXSAHM-11	0.40 pg/ml
IL-10	Interleukin 10	LXSAHM-11	1.6 pg/ml
MPO	Myeloperoxidase	LXSAHM-03	26.2 pg/ml
OPG	Osteoprotegerin	LXSAHM-11	3.62 pg/ml
VEGF-A	Vascular endothelial growth factor A	LXSAHM-11	2.1 pg/ml
VCAM-1	Vascular Cell Adhesion Molecule 1	LXSAHM-11	238 pg/ml
Biomarkers in kits purchased from Merck Millipore, Billerica, MA, USA			
ApoA1	Apolipoprotein A1	HNDG1MAG-36K	0.022 ng/ml
CFH	Complement Factor H	HNDG1MAG-36K	0.037 ng/ml
CRP	C-reactive protein	HCVD3MAG-67K	0.004 ng/ml
Fibrinogen	Fibrinogen	HCVD3MAG-67K	0.004 ng/ml
NCAM	Neural cell adhesion molecule	HNDG3MAG-36K	4.81 pg/ml

The sensitivities of the biomarkers evaluated in reagent kits purchased from R&D Systems are available on the manufacturer's assay configuration webpage: https://www.rndsystems.com/luminex/analytes. The sensitivities of biomarkers evaluated in kits purchased from Merck Millipore are available in the minimum detectable concentration (MinDC) tables that are included in all of the manufacturer's kit protocols.

Statistical Analysis

Continuous data are presented as median and interquartile range (IQR) and categorical data as frequency and percentage. Differences in individual biomarker levels between spinal TB and mechanical back pain were investigated using the Mann-Whitney U test for data with non-parametric distribution. The utility of each biomarker to distinguish between groups was assessed using Receiver Operating Characteristic (ROC) curves. Associations between participant characteristics and spinal TB and between participant characteristics and biomarker levels were investigated using a Mann-Whitney U test for continuous data and a Chi-squared or Fisher's exact test for categorical data. Associations between biomarker levels and spinal TB following adjustment for a participant characteristic were investigated using binomial logistic regression. The utility of combinations of biomarkers to identify spinal TB was investigated using General Discriminant Analysis (GDA) with leave-one-out cross validation. In addition to the GDA model generated from the current data, the performance of an existing pulmonary TB two-biomarker signature, CRP and CCL1 (26), was also evaluated. Finally, an exploratory Principal Component Analysis (PCA) was conducted to investigate linear combinations within the biomarker data. Data was log-transformed or winsorized as necessary prior to logistic regression, GDA and PCA. Analyses were conducted using Graphpad Prism (version 6.00, GraphPad Software, La Jolla, California, USA), jamovi (Version 1.1.9) (https://www.jamovi.org), Statistica (version 14, TIBCO Software Inc.) and the pROC (35) and UBbipl packages in R. Significance was accepted at $p < 0.05$.

RESULTS

Participant Characteristics

Twenty-eight patients with suspected spinal TB and 18 with mechanical back pain were recruited for the study. Two patients with suspected spinal TB were subsequently diagnosed with cancer and excluded. In addition, one patient with mechanical back pain was excluded after advanced imaging was suggestive for infection. The characteristics and clinical presentation of the remaining participants are shown in **Table 2**.

Thirteen participants with spinal TB and two participants with mechanical back pain were HIV-infected. Fourteen of these individuals were diagnosed with HIV previously and were on anti-retroviral treatment at the time of the study. One participant was diagnosed with HIV in the course of investigation for spinal TB and was not yet on antiretroviral treatment due to the need to first initiate TB treatment. CD4+ T-Lymphocyte and viral load values closest to the time of recruitment, up to a maximum of 7 months, are shown in **Table 2** [median time interval for CD4+ T-lymphocyte test *versus* recruitment = 0 months (IQR, 0 – 0.5 months), median time interval for viral load test *versus* recruitment = 0 months (IQR, 0 – 3.5 months)].

Spine Pathology Diagnosis

A diagnosis of spinal TB was bacteriologically confirmed in 18 (69%) of the 26 participants in the spinal TB group and clinically diagnosed in the remaining 8 (31%) participants. Clinical diagnoses were supported by a response to TB treatment in five participants whereas two participants passed away and one was lost to follow-up before treatment response could be evaluated. Of the 18 participants with bacteriologically confirmed spinal TB, Quantiferon tests were positive for 14 participants and not done for the remaining four. Of the eight participants with clinically-diagnosed spinal TB, Quantiferon tests were positive for five, negative in two and not done in one participant, respectively.

A diagnosis of mechanical back pain was confirmed by an orthopedic specialist with pathology including degenerative disc disease (n = 10), spondylosis (n = 2), stenosis (n = 2), herniated disc (n = 2) and fracture (n = 1). MRI findings contributed to the diagnosis in 13 of the 17 mechanical back pain participants whereas the remainder were diagnosed based on clinical presentation and X-ray findings alone.

Utility of Individual Biomarkers for Distinguishing Spinal TB

Median biomarker levels in participants with spinal TB and with mechanical back pain are shown in **Table 3**. Of the 19 biomarkers investigated, seven were significantly different between the groups, namely Fibrinogen, CRP, IFN-γ, NCAM, Ferritin, CCL1 and IL-10 ($p \leq 0.04$). Each of the afore-mentioned biomarkers was higher among those with spinal TB than among those with mechanical back pain. When evaluating the ROC plot for each biomarker, biomarkers with a significant difference between groups each had an AUC of ≥ 0.68 (**Table 3**). The four individual biomarkers with the highest diagnostic potential were Fibrinogen, CRP, IFN-γ and NCAM (AUC 0.88 – 0.99) (**Figure 1**).

Effect of Demographic and Clinical Characteristics on Individual Biomarkers

When the association between back pain group and participant characteristics was assessed, spinal TB was associated with younger age, HIV infection, shorter duration of back pain symptoms, constitutional symptoms and more severe pain on the Oswestry Disability Index ($p < 0.10$) (**Table 2**). These demographic and clinical characteristics were also associated with biomarker levels in some cases (**Table 4**). For example, Fibrinogen, CRP, IFN-γ, NCAM and Ferritin were associated with back pain duration ($p \leq 0.03$); and Fibrinogen, CRP and IFN-γ were associated with ODI ($p \leq 0.06$).

A preliminary investigation of the association between spinal TB and selected biomarkers following adjustment for the afore-mentioned participant characteristics is shown in **Table 5**. Logistic regression yielded very large odd's ratios in many cases due to large differences in certain biomarkers between back pain groups. Change between the unadjusted and adjusted odd's ratios suggests that participant characteristics may have influenced the association between back pain etiology and

TABLE 2 | Participant characteristics and clinical presentation.

	Spinal TB (n = 26)	Mechanical back pain (n = 17)	
Age, years, median [IQR]	48 [34-56]	53 [46-58]	p = 0.09
Gender, n (%)			p > 0.99
Male	12 (46)	7 (41)	
Female	14 (54)	10 (59)	
HIV status, n (%)			p = 0.02*
Positive	13 (50)	2 (12)	
Negative	11 (42)	12 (71)	
Unknown	2 (8)	3 (18)	
HIV positive, CD4+ T-lymphocytes, n (%)			
< 200 cells/μL	2 (15)	1 (50)	
200 – 499 cells/μL	3 (23)	0 (0)	
≥ 500 cells/μL	3 (23)	0 (0)	
Not available	5 (38)	1 (50)	
HIV positive, Log viral load, n (%)			
< 2.0 log copies/mL	7 (54)	1 (50)	
2.0 – 3.9 log copies/mL	2 (15)	0 (0)	
4.0 – 5.0	2 (15)	1 (50)	
Not available	2 (15)	0 (0)	
TB history, n (%)			p = 0.31
Previous TB	9 (35)	3 (18)	
No previous TB	17 (65)	14 (82)	
QuantiFERON test, n (%)			p = 0.02†
Positive	19 (73)	8 (47)	
Negative	2 (8)	7 (41)	
Indeterminate	1 (4)	1 (6)	
Not done	4 (15)	1 (6)	
Back pain duration, months, median [IQR]	6 [3-10]	24 [15-48]	p < 0.01
Constitutional symptoms‡, n (%)			p < 0.01
Yes	15 (58)	2 (12)	
No	11 (42)	15 (88)	
Concurrent pulmonary TB, n (%)			p = 0.51
Yes	2 (8)	0 (0)	
No	24 (92)	17 (100)	
Oswestry Disability Index category n (%)			p < 0.01
Minimal or moderate disability	3 (12)	10 (59)	
Severe disability or crippled	10 (38)	5 (29)	
Bed bound	13 (50)	2 (12)	

*TB, tuberculosis; IQR, inter-quartile range. Continuous variables were compared using a Mann-Whitney U test and categorical variables were compared using a Chi-squared or Fisher's exact test. *HIV positive vs. negative or unknown, †QFN positive vs QFN negative, ‡Constitutional symptoms include fever, weight loss and malaise.*

biomarkers. Nevertheless, Fibrinogen, CRP, IFN-γ, NCAM, Ferritin and CCL1 all remained significantly associated with spinal TB following adjustment for age, back pain duration, constitutional symptoms or ODI.

In a limited subgroup analysis, the differences in Fibrinogen, CRP, IFN-γ, NCAM and Ferritin concentrations between the spinal TB and mechanical back pain groups remained significant when including only individuals confirmed as HIV uninfected (spinal TB n = 11, mechanical back pain n = 12) and individuals without constitutional symptoms (spinal TB n = 11, mechanical back pain n = 15) (**Table 6**). Similarly, these differences remained significant when including only individuals ≥ 44 years of age, a cut-off which corresponded to the minimum age in the mechanical back pain group and allowed for a comparable age range between groups (spinal TB, n = 15, median age 54 years, IQR 49-64 years; mechanical back pain, n = 17, median age 53 years, IQR 46-57 years). In contrast, differences in CCL1 and IL-10 were no longer significant in most of these sub-analyses (**Table 6**)

Utility of Multi-Biomarker Signatures for Distinguishing Spinal TB

When evaluating combinations of biomarkers to diagnose spinal TB, GDA modeling identified a five-biomarker signature consisting of CRP, NCAM, Ferritin, CXCL8 and GDF-15 for optimal differentiation of spinal TB *versus* mechanical back pain (**Figure 2**). All biomarkers contributed significantly to the model at p < 0.01. The five-biomarker signature had sensitivity of 100% (95% C.I. 89 – 100%), a specificity of 100% (95% C.I. 84–100%) and a ROC plot AUC of 1.00 (95% C.I. 1.00-1.00). It correctly classified all 43 participants in both re-substitution classification and leave-one-out cross validation analyses. When reviewing the best 20 five-biomarker GDA models, CRP and NCAM appeared in all 20 models, IFN-γ in 17 models and VEGF-A in 10 models. All other biomarkers appeared in ≤ 7 of the 20 best models (**Figure 2**).

For the existing two-biomarker adult pulmonary TB-derived signature, CRP contributed significantly to the model (p < 0.001) but CCL1 did not (p = 0.91). The signature had a sensitivity of

TABLE 3 | Biomarker levels and utility of individual biomarkers to distinguish between spinal TB and mechanical back pain.

	Spinal TB	Mechanical back pain	p-value	AUC (95% C.I.)	AUC p-value	Sensitivity (95% C.I.)	Specificity (95% C.I.)
Fibrinogen	9120 [5729 – 16537]	1688 [1217 – 2155]	<0.001	0.99 (0.96 – 1.00)	<0.001	100.0 (89.1 – 100.0)	94.1 (71.3 – 99.9)
CRP	384770 [133567 – 517455]	11310 [6176 – 32819]	<0.001	0.95 (0.87 – 1.00)	<0.001	88.5 (69.8 – 97.6)	94.1 (71.3 – 99.9)
IFN-γ	27 [16 – 49]	0.00 [0.00 – 0.00]	<0.001	0.92 (0.83 – 1.00)	<0.001	92.3 (74.9 – 99.1)	94.1 (71.3 – 99.9)
NCAM	368555 [301809 – 447342]	217416 [189118 – 271548]	<0.001	0.88 (0.78 – 0.98)	<0.001	76.9 (56.4 – 91.0)	88.2 (63.6 – 98.5)
Ferritin	252060 [164326 – 1.32e+6]	105944 [50653 – 172244]	0.002	0.78 (0.64 – 0.92)	<0.001	69.2 (48.2 – 85.7)	82.4 (56.6 – 96.2)
CCL1	10 [7 – 20]	6 [5 – 9]	0.01	0.74 (0.59 – 0.90)	<0.001	73.1 (52.2 – 88.4)	64.7 (38.3 – 85.8)
IL-10	0.41 [0.00 – 1.78]	0.00 [0.00 – 0.00]	0.04	0.68 (0.53 – 0.82)	0.02	57.7 (36.9 – 76.6)	76.5 (50.1 – 93.2)
GDF-15	1054 [491 – 1396]	653 [416 – 853]	0.12	0.64 (0.48 – 0.81)	0.06	61.5 (40.6 – 79.8)	70.6 (44.0 – 89.7)
CXCL10	50 [30 – 61]	35 [28 – 46]	0.15	0.63 (0.46 – 0.80)	0.08	61.5 (40.6 – 79.8)	70.6 (44.0 – 89.7)
VEGF-A	192 [79 – 359]	143 [85 – 186]	0.15	0.63 (0.46 – 0.80)	0.07	50.0 (29.9 – 70.1)	88.2 (63.6 – 98.5)
CXCL9	1933 [1341 – 2949]	1575 [1408 – 1736]	0.20	0.62 (0.44 – 0.79)	0.10	57.7 (36.9 – 76.6)	88.2 (63.6 – 98.5)
Factor D	3.71e+6 [3.11e+6 – 4.31e+6]	4.17e+6 [3.59e+6 – 4.60e+6]	0.20	0.62 (0.44 – 0.79)	0.10	58.8 (32.9 – 81.6)	65.4 (44.3 – 82.8)
VCAM-1	1.05e+6 [759996 – 1.46e+6]	911522 [780792 – 1.11e+6]	0.22	0.61 (0.44 – 0.79)	0.11	53.8 (33.4 – 73.4)	70.6 (44.0 – 89.7)
ApoAI	243608 [218886 – 277761]	271259 [243145 – 308291]	0.23	0.61 (0.43 – 0.80)	0.11	70.6 (44.0 – 89.7)	53.8 (33.4 – 73.4)
MPO	481671 [254725 – 691086]	327798 [249070 – 502977]	0.30	0.60 (0.43 – 0.77)	0.15	57.7 (36.9 – 76.6)	70.6 (44.0 – 89.7)
ICAM-1	424590 [307328 – 603615]	347445 [241430 – 451406]	0.30	0.60 (0.41 – 0.78)	0.15	57.7 (36.9 – 76.6)	70.6 (44.0 – 89.7)
CXCL8	10 [6 – 17]	17 [8 – 23]	0.31	0.59 (0.41 – 0.78)	0.16	64.7 (38.3 – 85.8)	61.5 (40.6 – 79.8)
OPG	940 [759 – 1258]	1070 [870 – 1295]	0.53	0.56 (0.38 – 0.74)	0.27	53.8 (26.6 – 66.6)	64.7 (14.2 – 61.7)
CFH	484400 [463443 – 535822]	477628 [460687 – 544841]	0.89	0.51 (0.33 – 0.70)	0.45	52.9 (23.0 – 72.2)	61.5 (20.2 – 59.4)

TB, tuberculosis; AUC, area under the receiver operator characteristic plot; 95% C.I., 95% confidence interval. Biomarkers are ordered from highest to lowest AUC. Data are expressed as median and interquartile range with between-group differences assessed using a Mann-Whitney U test. CRP, Fibrinogen, ApoA1 and CFH measured in ng/ml and other biomarkers are measured in pg/ml.

92% (95% C.I. 75–99%), a specificity of 88% (95% C.I. 64–99%) and an AUC of 0.95 (95% C.I. 0.87-1.00)(**Figure 2**). It correctly classified 24 (92%) and 23 (89%) participants with spinal TB in the re-substitution classification and leave-one-out cross validation, respectively.

Principal Component Analysis

To further assess relationships between biomarkers and the two back pain groups, a PCA was performed. A biplot of the first two principal components (PC) identified from the biomarker data is presented in **Figure 3**. PCA generally produced a separation of spinal TB and mechanical back pain scores, although the total variation explained by PC 1 and PC 2 was relatively low at 39%. Fibrinogen and CRP, two of the biomarkers that showed the most potential as individual biomarkers for discriminating between spinal TB and mechanical back pain, were closely correlated and contributed variance to both PC 1 and PC 2. The concentrations of these biomarkers were higher in spinal TB than in mechanical back pain group and they appeared to have the best utility for discriminating between the spinal TB and mechanical back pain groupings. CXCL9, VEGF-A and CCL1 were also closely related and these biomarkers showed a positive association with PC 1. OPG, CXCL8 and CXCL10 showed a negative association with PC 2. PC 1 and PC 2 explained 28% and 11% of variation in the data, respectively.

DISCUSSION

The current study found that patients with spinal TB had experienced symptoms for several months at the time of diagnosis, in keeping with previous reports (7, 8, 12). Furthermore, the study was one of the few (29) to highlight the functional impact of spinal TB with 88% of patients reporting severe disability as a result of the associated back pain. These observations support the need for interventions that facilitate

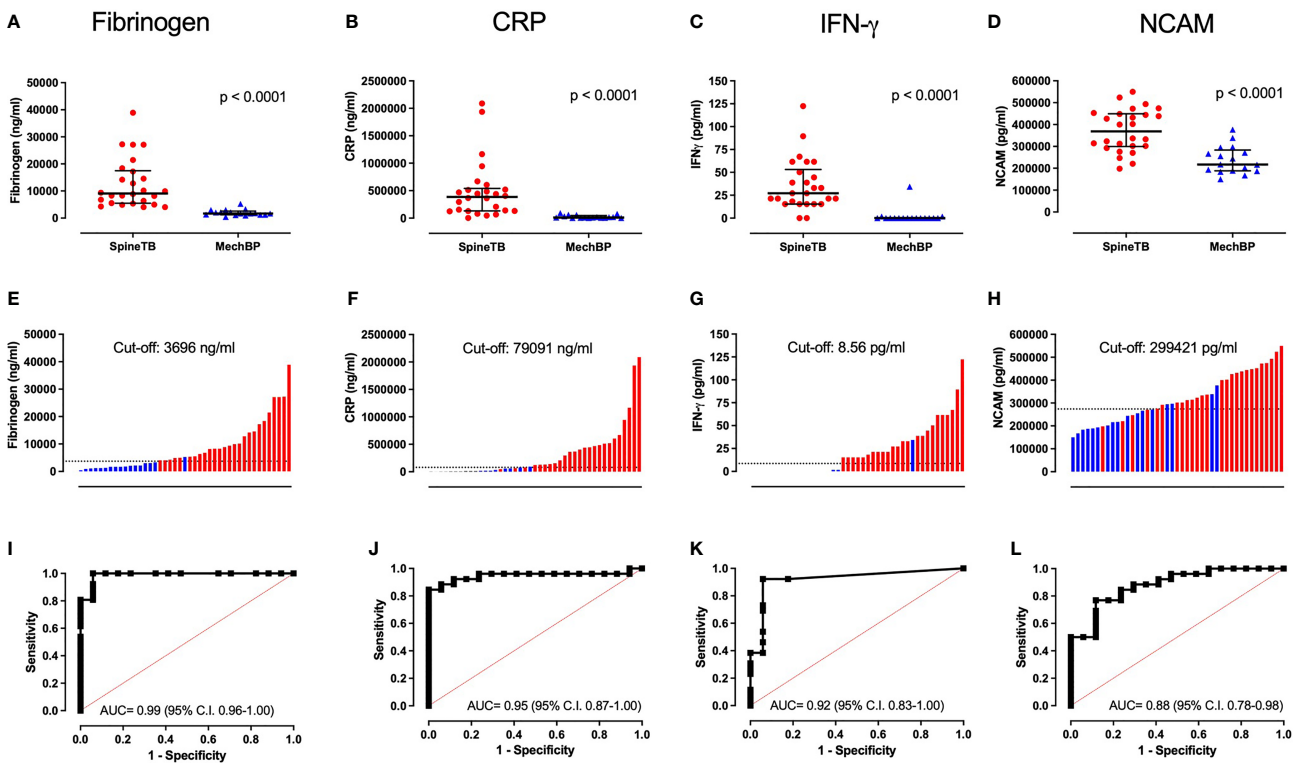

FIGURE 1 | Distribution of individual biomarker levels in spinal TB and mechanical back pain and the utility of biomarkers to distinguish spinal TB. **(A–D)** show the median and interquartile range of biomarkers within each group and the p-value of a Mann-Whitney U test; **(E–H)** show the rank order of individual biomarker levels when combining the groups with red bars representing spinal TB, blue bars representing mechanical back pain and the dashed line representing the cut-off biomarker value for optimal sensitivity and specificity; **(I–L)** show Receiver Operating Characteristic plots along with the Area Under the Curve (AUC) and associated 95% confidence interval. Representative plots for Fibrinogen, CRP, NCAM and IFN-γ are shown, each of which had an AUC ≥ 0.88.

earlier diagnosis of spinal TB, including the potential role of biomarker-based screening.

When investigating the utility of individual biomarkers, Fibrinogen, CRP, IFN-γ, NCAM and Ferritin showed strong potential to distinguish spinal TB from mechanical back pain (AUC ≥ 0.78). Furthermore these biomarkers generally had similar or slightly improved discriminative utility when restricting the analysis to those without HIV infection, those without constitutional symptoms, or those of a comparable age range. Overall, Fibrinogen and CRP were the most promising individual markers, which is notable in that Fibrinogen is one of the major determinants of ESR (15). ESR and CRP are existing screening measures for a potential infective etiology in back pain with levels typically elevated in spinal TB and normal in chronic mechanical back pain (36). ESR was not assessed in the current study and it is unclear whether Fibrinogen has higher discriminative potential than this conventional measure. Nevertheless, the study demonstrated that conventional marker

TABLE 4 | Association between selected biomarkers and demographic or clinical variables.

	Age		HIV status		QFN result		Back pain duration		Constitutional symptoms		ODI category	
	r^s	p-value	MW	p-value	MW	p-value	r^s	p-value	MW	p-value	χ^2	p-value
Fibrinogen	-0.16	0.32	138	0.30	54	0.01*	-0.55	<0.001*	96	0.002*	10.0	0.04*
CRP	-0.11	0.47	128	0.19	67	0.05	-0.57	<0.001*	116	0.01*	9.10	0.06
IFN-γ	-0.08	0.63	112	0.06	56	0.01*	-0.49	<0.001*	158	0.11	9.99	0.04*
NCAM	-0.28	0.07	139	0.32	60	0.03*	-0.43	0.004*	85	<0.001*	2.92	0.57
Ferritin	0.27	0.09	171	0.98	91	0.27	-0.33	0.03*	179	0.30	4.09	0.39
CCL1	-0.28	0.07	103	0.04*	83	0.17	-0.14	0.37	162	0.15	3.48	0.48
IL-10	-0.21	0.18	125	0.13	93	0.25	-0.19	0.22	200	0.58	1.98	0.74

*r^s, Spearman's rho; MW, Mann-Whitney U statistic; χ^2, Kruskal-Wallis statistic. Demographic and clinical variables were selected based on a difference between back pain groups at p < 0.10. The association between biomarkers and these variables was investigated as follows: Spearman's correlation for continuous variables age and back pain duration; Mann-Whitney U test for binomial variables HIV status, quantiferon (QFN) result and constitutional symptoms; Kruskal-Wallis test for categorical variable Oswestry Disability Index (ODI) category. Only confirmed results were included in the HIV and QFN analyses (n = 15 HIV positive, n = 23 HIV negative) (n = 27 QFN positive, n = 9 QFN negative). *Significant at p < 0.05.*

TABLE 5 | Association between spinal tuberculosis and selected biomarkers following adjustment for a demographic or clinical variable.

Log_{10} biomarker levels	Unadjusted		Age-adjusted		HIV-adjusted		Back pain duration-adjusted		Constitutional symptoms-adjusted		ODI category-adjusted	
	OR	p-value	OR	p-value	OR	p-value	OR	p-value	OR	p-value	OR	p-value
Fibrinogen	2.3×10^8	0.03*	1.5×10^8	0.04*	3.2×10^{205}	1.00	5.9×10^6	0.04*	2.8×10^8	0.03*	6.7×10^7	0.03*
CRP	35.5	<0.001*	499.2	0.01*	21.2	0.003*	13.8	0.01*	28.8	0.001*	24.8	0.002*
IFN-γ	35.0	<0.001*	36.6	<0.001*	273.5	0.04*	32.8	0.002*	32.0	<0.001*	29.0	<0.001*
NCAM	652891	<0.001*	367153.6	0.001*	405421.5	0.002*	387875.9	0.01*	378429.1	0.003*	9.6×10^6	0.002*
Ferritin	7.5	0.01*	26.2	0.001*	19.0	0.01*	12.5	0.02*	7.8	0.01*	7.5	0.02*
CCL	13.7	0.02*	11.4	0.04*	7.5	0.08	52.7	0.02*	9.9	0.04*	19.7	0.02*
IL-10	1.6	0.70	1.2	0.87	0.9	0.91	3.2	0.50	2.3	0.52	3.3	0.39

*Associations were investigated using binomial logistic regression with mechanical back pain as the dependent variable reference. Only one demographic or clinical variable was included along with the biomarker in each regression analysis. *Significant at $p < 0.05$*

CRP remained one of the most useful individual markers for distinguishing spinal TB from mechanical back pain despite the range of novel biomarkers investigated.

Although Fibrinogen and CRP performed well when differentiating between the current participants, it is likely that these biomarkers would have lower specificity for spinal TB within a primary care context. Furthermore, they may have lower levels earlier in the disease process or remain normal in a proportion of patients with spinal TB (12). Thus, use of a multi-biomarker signature would be expected to improve diagnostic accuracy and help to inform clinical decision-making among a more heterogeneous patient population. The current study identified a five-biomarker signature (CRP, NCAM, Ferritin, CXCL8 and GDF-15) which was able to correctly classify all participants regardless of HIV status. Nevertheless, the ability of this biomarker signature to identify spinal TB requires further validation, including prospective studies in primary care settings, with inclusion of any patient with chronic back pain, regardless of subsequently elucidated etiologies. Future studies should also consider further investigation of biomarkers that were not included in the best-performing biomarker signature, yet appeared in many of the top 20 GDA models. Examples of this include IFN-γ and VEGF-A, which appeared in 17 and 10 of the best 20 models, respectively. PCA explained a relatively low amount of variation in biomarker levels in the current study, suggesting limited potential for linear combinations of biomarkers in this context. Nevertheless, principal component findings supported further investigation

TABLE 6 | Biomarker levels and utility of individual biomarkers to distinguish between spinal TB and mechanical back pain in subgroups with no HIV infection, no constitutional symptoms or age 44 years and older.

	HIV uninfected				No constitutional symptoms				Age ≥ 44 years old			
	Spinal TB (n = 11)	Mech BP (n = 12)	p-value	AUC (95% C.I.)	Spinal TB (n = 11)	Mech BP (n = 15)	p-value	AUC (95% C.I.)	Spinal TB (n = 15)	Mech BP (n = 17)	p-value	AUC (95% C.I.)
Fibrinogen	12808 [9650 – 17798]	1688 [1184– 2992]	<0.001	1.00 (1.00–1.00)	8172 [4873 – 2144]	1688 [1217 – 2155]	<0.001	0.98 (0.94 – 1.00)	8882 [7468 – 16483]	1688 [1217 – 2155]	<0.001	0.99 (0.97–1.00)
CRP	445847 [168249 – 775863]	10829 [6046 – 22957]	<0.001	0.99 (0.97–1.00)	295179 [131666 – 469889]	11310 [5655 – 32819]	<0.001	0.98 (0.93 – 1.00)	361329 [142901 – 457868]	11310 [6176 – 32819]	<0.001	1.00 (0.98 – 1.00)
IFN-γ	27.2 [21.3– 33.0]	0.0 [0.0 – 0.0]	<0.001	0.95 (0.84–1.00)	33.0 [18.3 – 67.2]	0.0 [0.0 – 0.0]	<0.001	0.92 (0.79 – 1.00)	33.0 [18.3– 55.9]	0.0 [0.0 – 0.0]	<0.001	0.93 (0.83 – 1.00)
NCAM	402549 [292602 – 446360]	236484 [198490 – 295414]	0.01	0.83 (0.66–1.00)	301809 [275984 – 336740]	217416 [187371–294874]	0.01	0.81 (0.65 – 0.98)	312734 [273354 – 435458]	217416 [189118 – 271548]	0.001	0.82 (0.68 – 0.97)
Ferritin	397594 [217407 – 1.32e+6]	84557 [47554 – 144536]	0.002	0.88 (0.73–1.00)	460335 [174201 – 1315500]	70903 [38257 – 179191]	0.01	0.79 (0.60 – 0.99)	1.32e+6 [254472 – 1.32e+6]	105944 [50653 – 172244]	<0.001	0.89 (0.78 – 1.00)
CCL1	9.3 [6.1– 18.1]	6.2 [4.1– 9.2]	0.17	0.67 (0.45–0.90)	11.5 [6.4 – 19.8]	6.0 [2.6 – 8.5]	0.04	0.74 (0.54 – 0.93)	8.4 [6.1– 12.3]	6.0 [4.8– 8.5]	0.11	0.67 (0.48 – 0.86)
IL-10	0.3 [0.0 – 0.9]	0.0 [0.0 – 0.1]	0.21	0.64 (0.40–0.88)	0.2 [0.0 – 2.0]	0.0 [0.0 – 0.3]	0.13	0.66 (0.43 – 0.88)	0.0 [0.0 – 0.6]	0.0 [0.0 – 0.0]	0.37	0.58 (0.38 – 0.78)

TB, tuberculosis; Mech BP, Mechanical Back Pain; AUC, area under curve; C.I, confidence interval. Subgroup analyses are presented only for biomarkers that were significantly different between spinal TB and mech BP at the whole-group level. Data are presented as median and interquartile range of biomarker levels with between subgroup differences assessed using a Mann-Whitney U test.

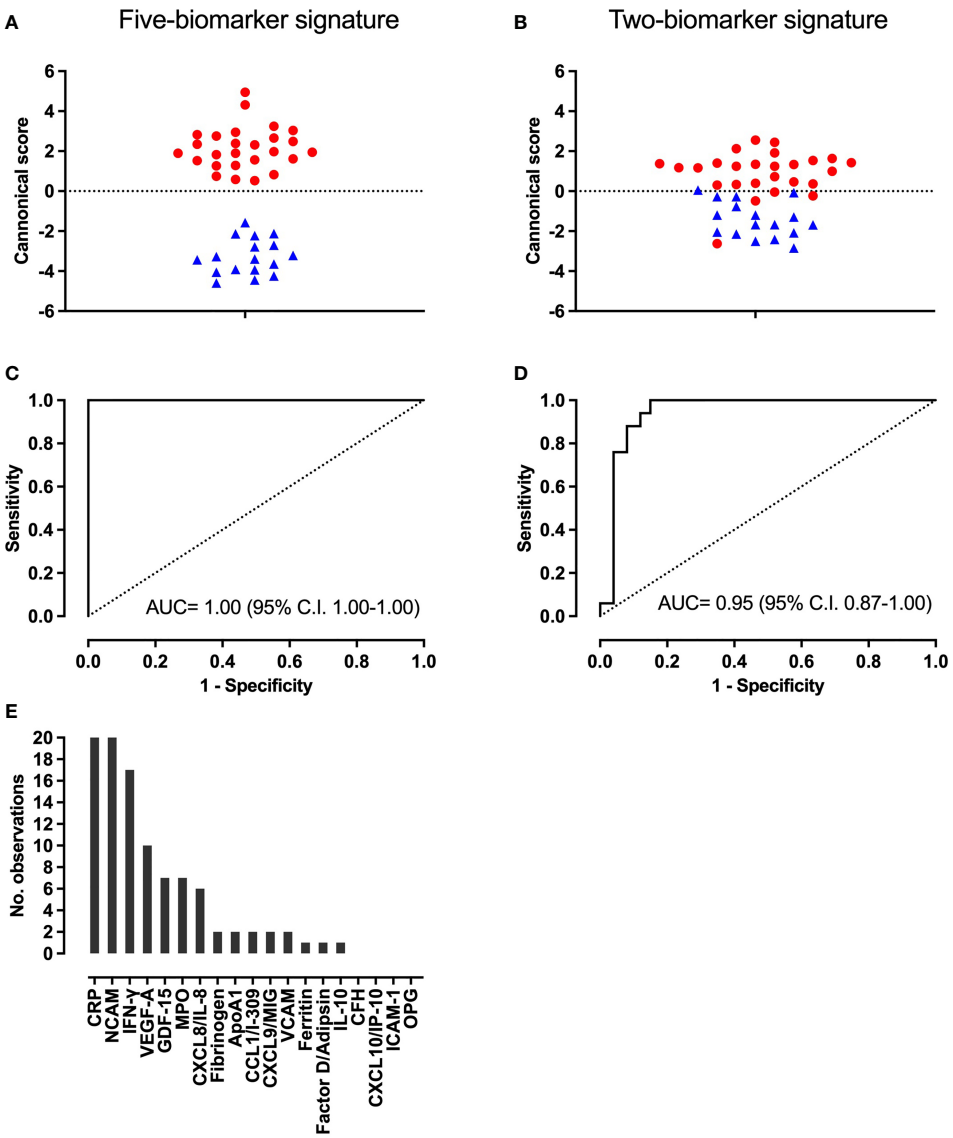

FIGURE 2 | Accuracy of the new five-biomarker signature (CRP, NCAM, Ferritin, CXCL8 and GDF-15) and existing two-marker biosignature (CRP and CCL1) for distinguishing spinal TB and mechanical back pain. **(A, B)** Plot of canonical scores derived from each model, **(C, D)**, Receiver Operating Characteristic plots showing the accuracy of each model and **(E)** Number of times each biomarker appeared in the top 20 five-biomarker General Discriminant Analysis models for discriminating between spinal TB and mechanical back pain. In panels **(A, B)**, red dots and blue triangles represent the scores of individuals with spinal TB and mechanical back pain, respectively.

of several of the biomarkers identified through other analyses, including Fibrinogen, CRP, VEGF-A and CCL1.

Validation of the current findings in larger studies is particularly important in light of differences in HIV infection, back pain duration and back pain severity between the spinal TB and mechanical back pain groups. Most of the top-performing biomarkers remained significantly associated with spinal TB following adjustment for participant characteristics. However, the sample size and distribution of the data were notable limitations in these analyses. For example, although biomarker associations remained significant in the subgroup without HIV infection, the study was not able to adequately explore the influence of HIV infection on biomarker utility as only two individuals in the mechanical back pain group were HIV infected. Spinal TB is a comparatively uncommon form of TB and it was necessary to conduct the study at a tertiary hospital in order to optimize recruitment. However, limited resources for this exploratory research meant that it was necessary to recruit participants with mechanical back pain from the same setting. As observed in the study, patients with spinal TB may have developed very severe pain by the time of referral to a tertiary hospital and patients with mechanical back pain may have had

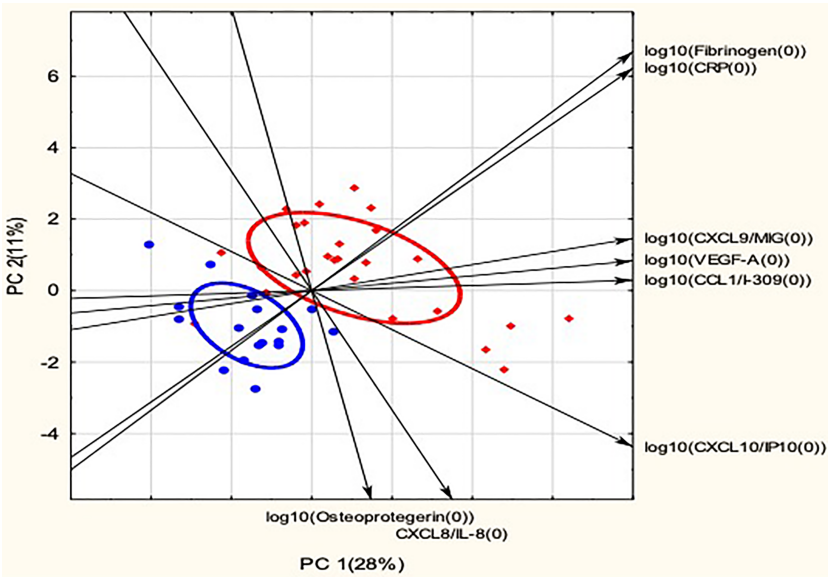

FIGURE 3 | Principal Component Analysis of biomarker distributions in spinal TB and mechanical back pain. Red diamonds indicate scores for spinal TB and blue dots indicate scores for mechanical back pain. Ellipses indicate 50% confidence ellipses for spinal TB and mechanical back pain, respectively. Arrows indicate the direction of increasing biomarker levels. Only biomarkers with $R^2 > 0.40$ for principal components 1 and 2 are shown. PC, Principal Component with the percentage of variation explained by the principal component shown in brackets.

pain for a long duration before being referred to a tertiary hospital. Overlap in patient characteristics between the different back pain etiologies would be expected to be larger at primary care level, where biomarker-based screening would provide most benefit.

Previous studies suggest differential cytokine responses in different types of TB (37) and the optimal diagnostic biomarker signatures may likewise vary according to type of TB disease (33). Nevertheless, a test with utility across different types of TB would have practical advantages and it is interesting to compare findings when the inclusion of the same biomarkers allows (33). Two previous studies in pulmonary TB included several of the same biomarkers as in the current study and also used the same analysis platform (22, 27). As with the current study, CRP (22, 27), NCAM (27), IFN-γ (22), Ferritin (27) and Fibrinogen (22) were among the top-performing individual biomarkers in one or both of these previous studies, depending on the other biomarkers included. However, whereas both Fibrinogen and CRP had high diagnostic utility in the current study, Fibrinogen appeared to have lower diagnostic utility in pulmonary TB with one study reporting an AUC of 0.70 for Fibrinogen *versus* 0.86 for CRP (22). Conversely, it was noted that CXCL10 appeared to have higher diagnostic utility in pulmonary TB than in the current study. CXCL10 has received considerable attention as a diagnostic marker for pulmonary TB with an AUC of 0.78 and 0.83 in the afore-mentioned studies and an overall AUC of 0.93 in a recent meta-analysis (38). However, in the current study, CXCL10 was not significantly different between the spinal TB and mechanical back pain groups and had an AUC of only 0.63. Similarly, CXCL10 levels showed poor utility to discriminate between back pain etiology groupings in the PCA. Variation in biomarker utility between different types of TB may be related to factors such as tissue-specific variation in immunopathogenesis and compartmentalization of immune cells (39).

When considering combinations of biomarkers from previous studies, the existing pulmonary TB biosignature of CRP and CCL1 performed well in the current study, achieving an AUC of 0.95 compared to 0.90 in the original test set of suspected pulmonary TB (26). While this is somewhat encouraging, it was noted that CCL1 did not contribute significantly to the model in the current study and the model AUC was the same as that of CRP alone. In the current study, CCL1 was no longer significantly different between spinal TB and mechanical back pain in subgroups without HIV infection or of similar age range and it is possible that this analyte has somewhat less utility for distinguishing spinal TB than pulmonary TB. While this observation requires investigation in larger studies, it makes the point that testing existing biomarker signatures alongside optimal combinations can provide helpful additional insights (40)

The current study had several limitations including the small sample size and the case-control study design. As previously discussed, the study was also limited by differences in certain participant characteristics, such as HIV infection, and was not able to adequately explore the effect of these characteristics on biomarker utility. Due to the exploratory nature of the study, we chose not to correct for multiple testing. The rationale was that such corrections may risk relevant biomarker associations being missed and not carried forward into future studies. Although many of the current biomarker findings are in keeping with

previous literature, there remains a risk of false positive results and this constitutes a further limitation of our study.

Although the findings are preliminary, biomarkers that showed potential, either individually or through inclusion in the biomarker models, could be considered candidates for further investigation in larger studies. Given that back pain originating from spinal TB may not always be accompanied by constitutional symptoms, such future studies could consider recruiting all individuals presenting with more than 6 weeks of back pain in a TB endemic setting. These individuals could then be monitored prospectively to detect those diagnosed with spinal TB and those confirmed with an alternative diagnosis. Such studies should also assess the influence of HIV status, including the severity of HIV/AIDS disease, on the diagnostic accuracy of the biomarkers.

In conclusion, the current case-control study evaluated a novel selection of biomarkers for distinguishing spinal TB from mechanical back pain and identified both individual biomarkers and a five-biomarker signature as candidates in this regard. These preliminary findings require validation in larger studies, including prospective cohort studies to assess the accuracy of candidate biomarkers among patients presenting to primary care health facilities with chronic back pain.

DATA AVAILABILITY STATEMENT

The raw data supporting the conclusions of this article will be made available by the authors, without undue reservation.

ETHICS STATEMENT

This study involving human participants was reviewed and approved by the Health Research Ethics Committee of Stellenbosch University and by hospital management. The patients provided written informed consent to participate in the study.

AUTHOR CONTRIBUTIONS

TM: Conceptualization, investigation, formal analysis, project administration, visualization, and writing—original draft. JD: Methodology, investigation, and writing—review and editing. GW: Methodology, resources, and writing—review and editing. CB: Investigation and writing—review and editing. JT: Resources, supervision, and writing—review and editing. RL: Supervision and writing—review and editing. NC: Methodology, investigation, resources, and writing—review and editing. All authors contributed to the article and approved the submitted version.

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Longitudinal Variations of *M. tuberculosis*-Induced IFN-γ Responses in HIV-Negative Pregnant Women Exposed to Tuberculosis

Paulo Ranaivomanana[1], *Rila Ratovoson*[2], *Crisca Razafimahatratra*[2], *Arimanitra Razafimahefa*[1], *Jonathan Hoffmann*[3], *Perlinot Herindrainy*[2], *Julio Rakotonirina*[4] *and Niaina Rakotosamimanana*[1]*

[1] Mycobacteria Unit, Institut Pasteur de Madagascar, Antananarivo, Madagascar, [2] Epidemiology Unit, Institut Pasteur de Madagascar, Antananarivo, Madagascar, [3] Medical and Scientific Department, Fondation Mérieux, Lyon, France, [4] Centre Hospitalier Universitaire de Soins et Santé Publique Analakely (CHUSSPA), Antananarivo, Madagascar

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Novel N. Chegou,
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Francis Crick Institute, United Kingdom

***Correspondence:**
Niaina Rakotosamimanana
niaina@pasteur.mg

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Introduction: Pregnancy triggers an alteration of the immune functions and increases the risk of developing the active tuberculosis (TB) symptoms in exposed women. The effect of pregnancy on the *Mycobacterium tuberculosis*-specific immune responses used for most of the TB immunodiagnostic assays is not well documented. Here we investigated the changes in the *M. tuberculosis*-specific IFN-γ production in age-matched pregnant and non-pregnant women according to their TB exposition status.

Methods: We conducted a prospective cohort study on HIV-seronegative pregnant and non-pregnant women with compatible pulmonary TB symptoms addressed to TB healthcare facilities in Antananarivo, Madagascar. Active pulmonary TB was bacteriologically assessed with culture from sputum samples. Clinical data and blood samples were collected at inclusion and after 6 months of follow-up for each individual included. Whole blood samples were stimulated with QuantiFERON TB-Gold Plus (QFT-P) assay antigens. Plasma IFN-γ concentrations were then assessed by ELISA.

Results: A total of 284 women were investigated for the study including 209 pregnant women without confirmed TB (pNTB), 24 pregnant women with bacteriologically confirmed active TB (pATB), 16 non-pregnant women with active TB (ATB), and 35 non-pregnant healthy donors (HC). At inclusion, IFN-γ responses are lower in the pregnant women compared to their age-matched non-pregnant counterparts and independently of their TB status. Among the pregnant women, higher concentrations of *M. tuberculosis*-specific IFN-γ were observed in those exposed to TB, but with a lower magnitude in the active TB compared to the latently infected pregnant women ($p < 0.05$ with TB1 and $p < 0.01$ with TB2). After 6 months of follow-up, the *M. tuberculosis*-specific IFN-γ responses return to their baseline concentrations except for the pregnant women treated for TB for which none of the QFT-P positive reversed to negative (0%, 0/10) at the end of their TB treatment.

Conclusion: These results support the concept of specific immune priorities characterized by a concomitant reduction in inflammatory immunity during pregnancy and corroborate the important role of activating the *M. tuberculosis*-specific immune responses to control the infection when the pregnant women are exposed to the pathogen.

Keywords: tuberculosis, interferon-gamma release assays (IGRA), pregnancy, human whole blood, QuantiFERON-TB Gold Plus®

INTRODUCTION

Tuberculosis (TB) remains a public health threat worldwide with an estimated 10 million notified cases and 1.4 million deaths every year (1). TB is an important cause of maternal mortality and morbidity globally, and the number of TB cases in women of reproductive age (15–49 years) is increasing (1). Particularly, TB in pregnancy was found to be associated with increased risks of pregnancy complications, prematurity, neonatal morbidity, low birth weight, and perinatal deaths (2, 3).

The physiological changes during pregnancy result in distinct immune responses that lead to an immunological tolerance toward the fetus characterized by downregulations of both the cell-mediated immunity and the production of Th1 cytokines and an upregulation of both the humoral immunity and the Th2 cytokine productions (4, 5). While this maternal immune tolerance protects the fetus from rejection and promote a healthy pregnancy outcome, it may increase the vulnerability against pathogens like TB as the cell-mediated immunity and the Th1 response play key roles in containing the *M. tuberculosis* infection (6, 7).

Interferon-gamma release assays (IGRAs) are immunoassays based on the measure of the production of IFN-γ produced after T cell *ex vivo* stimulation by *M. tuberculosis*-specific antigens also used to assess for a previous exposure or infection to *M. tuberculosis*. Recent studies on the IFN-γ response changes in *M. tuberculosis* latently infected pregnant women (pLTBI+) using IGRAs suggested a slight increase of the Th1 pro-inflammatory response to a concentration sufficient to contain the latent infection in these pregnant women (7–9 and 10). However, few data are available on this IFN-γ immune response status in pregnant women with active TB disease.

In the present study, we investigated the *M. tuberculosis*-specific immune response in pregnant women with active TB disease. Moreover, we assess the IFN-γ response changes postpartum and/or following a successful TB treatment and compare them to those from non-pregnant women with or without active TB disease.

METHODS

Study Design and Setting

Study participants were included from two studies: a cross-sectional prospective cohort study for non-pregnant women and one cohort survey for pregnant women conducted between April 2018 to August 2019 in primary healthcare facilities in the capital city of Antananarivo, Madagascar [as previously described in 11 and Chedid et al., 2012 (12)]. Pregnant women with TB-compatible symptoms defined as one or a combination of cough more than 2 weeks, hemoptysis, dyspnea, chest pain, weight loss, night sweats, loss of appetite, fever, and deterioration of general condition were invited to participate. Non-pregnant women with presumptive TB symptoms were also recruited if diagnosed with bacteriologically confirmed pulmonary TB (ATB) (see *Mycobacteriology* section for further detail).

To investigate the baseline responses for *M. tuberculosis* non-exposed individuals, age-matched community control non-pregnant women without symptoms or signs of TB and no known recent or sustained contact with TB cases (HC) were recruited at the post-exposure rabies center of Institute Pasteur de Madagascar (Antananarivo, Madagascar).

Patients with HIV (human immunodeficiency virus) or diabetes mellitus and participants under 15 years were excluded from the study. In downstream analyses, patients under immunocompromising treatment (corticosteroids, calcineurin inhibitors, biologics, or other chemotherapeutic agents) were excluded from the analyses.

Data Collection and Laboratory Procedures

Sociodemographic characteristics, TB history, pregnancy-related factors (i.e., gestational age, parity), Bacillus Calmette–Guérin (BCG) vaccinal status, comorbidities (HIV, hepatitis), and data on TB-presumptive clinical symptoms were collected using standardized questionnaires by trained nurses.

Mycobacteriology

Confirmed TB cases were defined as smear microscopy positive for AFB (Ziehl–Neelsen and/or Auramine staining) and Lowenstein–Jensen solid medium culture positive for *M. tuberculosis* from sputum samples.

Interferon-Gamma Assay

A 5-ml venous blood sample was drawn in a blood-collecting tube containing lithium heparin for each participant. The blood samples were transported to the laboratory within 4 h after blood sampling at the controlled temperature (22 ± 5°C), and a 1-ml sample was dispensed into each assay tube, followed by mixing by inversion, and incubated at 37°C for 16–20 h within 16 h after collection. After incubation, the tubes were centrifuged, and aliquots of the supernatants were stored at -20°C.

IFN-γ secretion was quantified using the QFT-P ELISA Kit (Qiagen) according to the manufacturer's instructions (13). Briefly, plasma samples were thawed at room temperature, and 50 µl of plasma was tested. Optical density results were compared to log-normalized values from freshly reconstituted IFN-γ kit standards. To account for potential immunomodulation phenomena, baseline IFN-γ concentration values (NIL tubes) were subtracted from antigen-stimulated IFN-γ values (MIT, TB1, TB2). The NIL tube contained no antigens and was used as a negative control. The TB1 and TB2 QFT-P tubes are coated with commercial *M. tuberculosis*-specific antigenic peptide pools. TB1 tubes contain two mycobacterial peptides, ESAT-6 (>15 aa) and CFP-10, which elicit specific immune responses from CD4+ T lymphocytes (13). TB2 tubes contain an additional commercial peptide pool designed to induce CD8+ T lymphocyte stimulation. MIT tubes are coated with commercial phytohemagglutinin-like bacterial antigens and were used as a positive control (13).

According to the kit's sensitivity range, the maximum for the IFN-γ concentration value was set at 10 IU/ml and negative values were rescaled to 0; the cutoff value for a positive test was 0.35 IU/ml.

Follow-Up Visits

Each participant except the HC group was followed up at inclusion (D0) and at 6 months (M6) where QFT-P tests were performed. Confirmed TB patients were put on Directly Observed Treatment (DOT) and received treatment according to standard WHO protocols (14). Briefly, for the TB-confirmed participants, the treatment consisted of 2 months of daily rifampicin, isoniazid, pyrazinamide, and ethambutol, followed by 4 months of daily rifampicin and isoniazid. The successful TB treatment course is assessed based on the resolution of clinical symptoms and sputum conversion to negativity (microscopy and culture) according to the Malagasy national and WHO guidelines. For the pregnant women, all maternal and fetal outcomes were collected including delivery.

Statistical Analysis

All data generated from the study were recorded in a REDCap® software database. GraphPad Prism version 8.0 was used for statistical analyses. The $\chi 2$ and Fisher exact tests were performed to compare categorical variables between groups, as appropriate. The Mann–Whitney test was used to analyze the magnitude differences of IFN-γ responses between groups. Pairwise comparisons of *M. tuberculosis*-triggered IFN-γ concentrations were performed between DO and M6 separately for the two antigens (TB1 and TB2) using the Wilcoxon matched-pair signed-rank test. A p-value <0.05 was considered statistically significant.

Ethical Considerations

All information about the study was explained in local language; written informed consent and completed questionnaires were obtained from all included participants. The methods were carried out in accordance with the approved guidelines. The 2 studies were approved by the Ethical Committee for Biomedical Research in Madagascar (reference number: 099 MSANP/CERBM 2018 and 103MSANP/CERBM 2017).

RESULTS

Study Participant Characteristics

A total of 284 women were included in the study including 209 pregnant women without bacteriologically confirmed TB (pNTB), 24 pregnant women with active TB (pATB), and 16 non-pregnant women with active TB (ATB) (**Table 1**). Moreover, thirty-five (35) non-pregnant healthy women donors (HD) were also recruited as controls. There was no significant difference in the age between pregnant and non-pregnant groups. Among pregnant women (pNTB and pATB), 91% of the studied population had been vaccinated with BCG at childhood without significant difference between pregnant and non-pregnant women (**Table 1**).

The clinical symptoms for each participant in pregnant women (pNTB and pATB) and ATB groups are described in **Table 2**. Cough was reported for all included pregnant women and the ATB group at inclusion (100%). One participant had HIV infection, and another had hepatitis. Both were excluded from analyses.

Concentrations of IFN-γ Production Upon *M. tuberculosis* Antigen Stimulation by Pregnancy Status

In order to study the impact of pregnancy on the IFN-γ production upon *M. tuberculosis* antigen stimulation, the plasma IFN-γ concentrations in response to TB1 and TB2 were measured with the QFT-P assay in pregnant and non-pregnant women within the active clinical TB subgroup: pATB vs. ATB; the non-confirmed-TB pregnant women with positive QFT-P (pLTBI+) vs. the healthy non-pregnant women with positive QFT-P (LTBI+); and the non confirmed-TB pregnant women with negative QFT-P (pLTBI-) vs. healthy non-pregnant women with negative QFT-P (LTBI-) (**Figure 1**).

A globally lower median concentration of IFN-γ in response to TB1 or TB2 was observed in the pregnant women of the different clinical subgroups compared to their non-pregnant counterparts. This lower IFN-γ concentration is particularly significant ($p < 0.0001$) in pLTBI- compared to LTBI- with a mean IFN-γ concentration of 0.04 vs. 0.34 IU/ml with TB1 and 0.05 vs. 0.34 IU/ml, with TB2). Among the women with positive QFT-P, this difference was significant after stimulations with TB1 (respectively with a mean IFN-γ of 2.04 vs. 4.05 IU/ml, $p < 0.05$ for pLTBI+ vs. LTBI+), while it was non-significant after stimulations with TB2, despite a lower IFN-γ concentration in the pregnant women after stimulation of both antigens (**Figures 1A, B**).

No statistically significant differences were observed when comparing the IFN-γ concentrations in the active TB group

TABLE 1 | Baseline characteristics of the study participants.

Characteristics	pNTB n = 202	pATB n = 24	ATB n = 16	p value
Age (mean SD)	26.2 (5.4)	25.7 (5.3)	28.8 (12.1)	>0.05
Gravidity				
1	70 (34.7)	11 (45.8)	–	0.55
2	39 (19.3)	4 (16.7)	–	
≥3	93 (46.0)	9 (37.5)	–	
Marital status				
Married	77 (38.1)	8 (34.8)	-	0.86
Free union	120 (59.4)	15 (65.2)	-	
Single	4 (2.0)	0 (0.0)	-	
Divorced/widowed	1 (0.5)	0 (0.0)	-	
Educational level				
No	9 (4.5)	0 (0.0)	0 (0.0)	0.80
Primary	56 (27.7)	5 (21.7)	6 (37.5)	
Secondary	112 (55.4)	15 (65.2)	8 (50.0)	
University	25 (12.4)	3 (13.0)	2 (12.5)	
Occupational status			n/a	
Unemployed	79 (39.1)	11 (47.8)	6 (37.5)	<0.0001
Manual work	120 (59.4)	11 (47.8)	7 (43.8)	
Work in an office	0 (0.0)	1 (4.3)	0(0.0)	
Student	3 (1.5)	0(0.0)	3 (18.8)	
TB contact history				
Yes	37 (18.3)	12 (50.0)	9 (56.1)	<0.0001
No	163 (80.7)	11 (45.8)	2 (12.5)	
Unknown	2 (1.0)	1 (4.2)	5 (31.3)	
TB treatment history				
Yes	12 (5.9)	3 (12.5)	3 (18.7)	0.10
No	190 (94.1)	21 (87.5)	13 (81.3)	
BCG vaccination				
Yes	185 (91.5)	21 (91.4)	14 (87.4)	0.97
No	7 (3.5)	1 (4.3)	1 (6.3)	
Unknown	10 (5.0)	1 (4.3)	1 (6.3)	
Smoking status				
Yes	2 (1.0)	0(0.0)	4 (25.0)	<0.0001
No	200 (99.0)	24 (100.0)	12 (75.0)	
Alcohol usage				
Yes	32 (15.8)	4 (16.7)	1 (6.3)	0.57
No	170 (84.2)	20 (83.3)	15 (93.7)	
HIV infection				
Yes	1 (0.5)	0(0.0)	0(0.0)	0.29
No	167 (82.7)	17 (73.9)	16 (100.0)	
Unknown	34 (16.8)	6 (26.1)	0(0.0)	
Hepatitis				
Yes	0(0.0)	1 (4.3)	0(0.0)	0.008
No	202 (100.0)	22 (95.7)	16 (100.0)	

TB, tuberculosis; pNTB, non-confirmed TB pregnant women; pATB, active TB-confirmed pregnant women; ATB, active TB-confirmed non pregnant women; BCG, Bacille de Calmette–Guerin; HIV, human immunodeficiency virus.

TABLE 2 | Description of presumptive TB clinical symptoms of the study participants.

Clinical symptoms	pNTB n = 204	pATB n = 23	ATB n = 15	p value
Cough	204 (100.0)	23 (100.0)	15 (100.0)	1.00
Fever	62 (30.4)	9 (39.1)	10 (66.7)	0.01
Hemoptysis	24 (11.8)	8 (34.8)	3 (20.0)	0.009
Weight loss	95 (46.6)	19 (82.6)	15 (100.0)	<0.001
Loss of appetite	83 (40.7)	16 (69.6)	-	0.01
Night swear	69 (33.8)	14 (60.9)	13 (86.7)	0.0002
Dyspnea	96 (47.1)	14 (60.9)	-	0.70
Chest pain	73 (35.8)	9 (39.1)	11 (73.3)	0.01
Other symptoms/comorbidities	24 (11.8)	1 (4.3)	3 (20.0)	0.32

pNTB, non-confirmed TB pregnant women; pATB, active TB-confirmed pregnant women; ATB, active TB-confirmed non pregnant women.

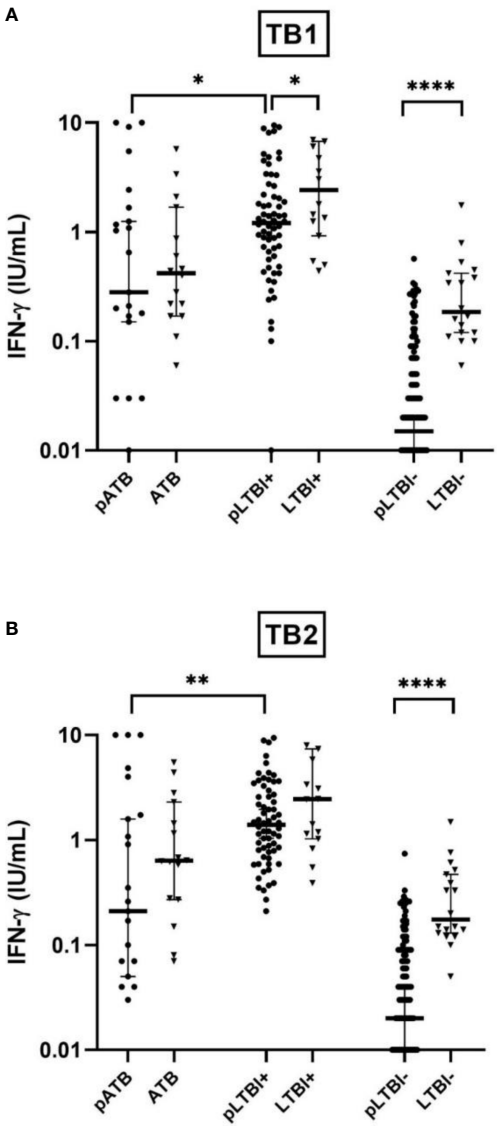

FIGURE 1 | Comparison of IFN-γ production response by QuantiFERON-TB Gold-Plus Assay with stimulation with TB1 **(A)** and TB2 **(B)** antigens between pregnant women (pATB, pLTBI-, and pLTBI+) and their non-pregnant counterparts (ATB, LTBI-, and LTBI+). Symbols indicate individual IFN-γ values for pregnant participants (black circles) and non-pregnant (black triangles). The solid lines indicate median and 95% CI. The Mann–Whitney U-test (two-tailed) comparison were calculated and shown as *$p < 0.05$, **$p < 0.01$, and ****$p < 0.0001$. pATB, TB-confirmed pregnant women; ATB, TB-confirmed non-pregnant women; pLTBI-, pregnant women with QFT-P negative response; pLTBI+, pregnant women with QFT-P positive response; LTBI-, healthy non-pregnant control with QFT-P negative response; LTBI+, healthy non-pregnant control with QFT-P positive response.

(pATB vs. ATB), despite lower concentrations of IFN-γ in the pregnant women in response to both TB1 and TB2 (**Figure 1**).

Finally, among the pregnant women, low IFN-γ magnitudes were observed in the pATB group compared to the pLTBI+ group ($p < 0.05$ with TB1 and $p < 0.01$ with TB2) (**Figure 1**).

Comparison of *M. tuberculosis* Antigen Triggered IFN-γ Concentrations After 6 Months Follow-Up (TB Treatment and Postpartum)

All the participants confirmed of active TB successfully completed their TB treatment course, based on resolution of clinical symptoms and sputum conversion to negativity. There was no occurrence of relapse or recurrence recorded. One hundred and twenty-three (123) pregnant women (105 pNTB and 18 pATB) and 15 ATB had a 6-month follow-up visit (M6) (**Figure 2**).

At M6, although not statistically significant, increased concentrations of IFN-γ were observed in the pLTBI- group after TB1 or TB2 stimulations compared to the concentrations measured at the inclusion (D0) (**Figures 2C, D**).

Among the pLTBI+, a decrease in IFN-γ concentrations in the pregnant women at M6 after stimulation with both antigens was observed compared to the concentrations measured at D0 and this difference was statistically significant with TB2 antigen stimulation ($p < 0.05$) (**Figure 2F**). No statistical differences in IFN-γ response magnitude were observed between measures at D0 and M6 for the pATB and ATB groups (**Figures 2A, B, G, H**).

QFT-Plus Assay Qualitative Evolution

After assigning the manufacturer threshold for qualitative positivity, a QFT-P positivity of 58.3% was observed in pATB, 34.4% in pNTB, 62.5% in ATB, and 45.7% in HC (**Table 3**).

Table 4 shows the QFT-P assay result regarding the characteristics of pregnant women groups. In univariate analysis, it was observed that older age ($p < 0.05$), previous TB treatment, and previous TB contact histories ($p < 0.01$) were associated with positive QFT-P response. No association on QFT-P result was observed regarding pregnancy-related factors (gestational age, parity, and gravidity) and pregnancy outcomes (preterm vs. full-term birth; caesarian vs. normal vaginal birth) (data not shown).

Figure 3 shows the conversion/reversion rates of QFT-P assay at M6. The conversion rates were respectively 20.3% (13/64), 25% (2/8), and 60% (3/5) for the pNTB, pATB, and ATB groups, respectively. The pNTB and ATB groups had reversion rates of respectively 31.7% (13/41) and 40% (4/10), respectively. Interestingly, all the 10 pregnant women in the pATB group who had a positive QFT-P test at inclusion remained with positive QFT-P at M6 giving a reversion rate of 0% (0/10) with similar concentrations of IFN-γ production magnitude at D0 and at the end of their TB treatment (**Figure 3**).

DISCUSSION

This study was performed to analyze the impacts of the immune system changes during the pregnancy that might contribute to the increased risk of morbidity and mortality associated with *M. tuberculosis* exposure. We observed a decreased IFN-γ production from whole blood T cell *ex vivo* stimulation with

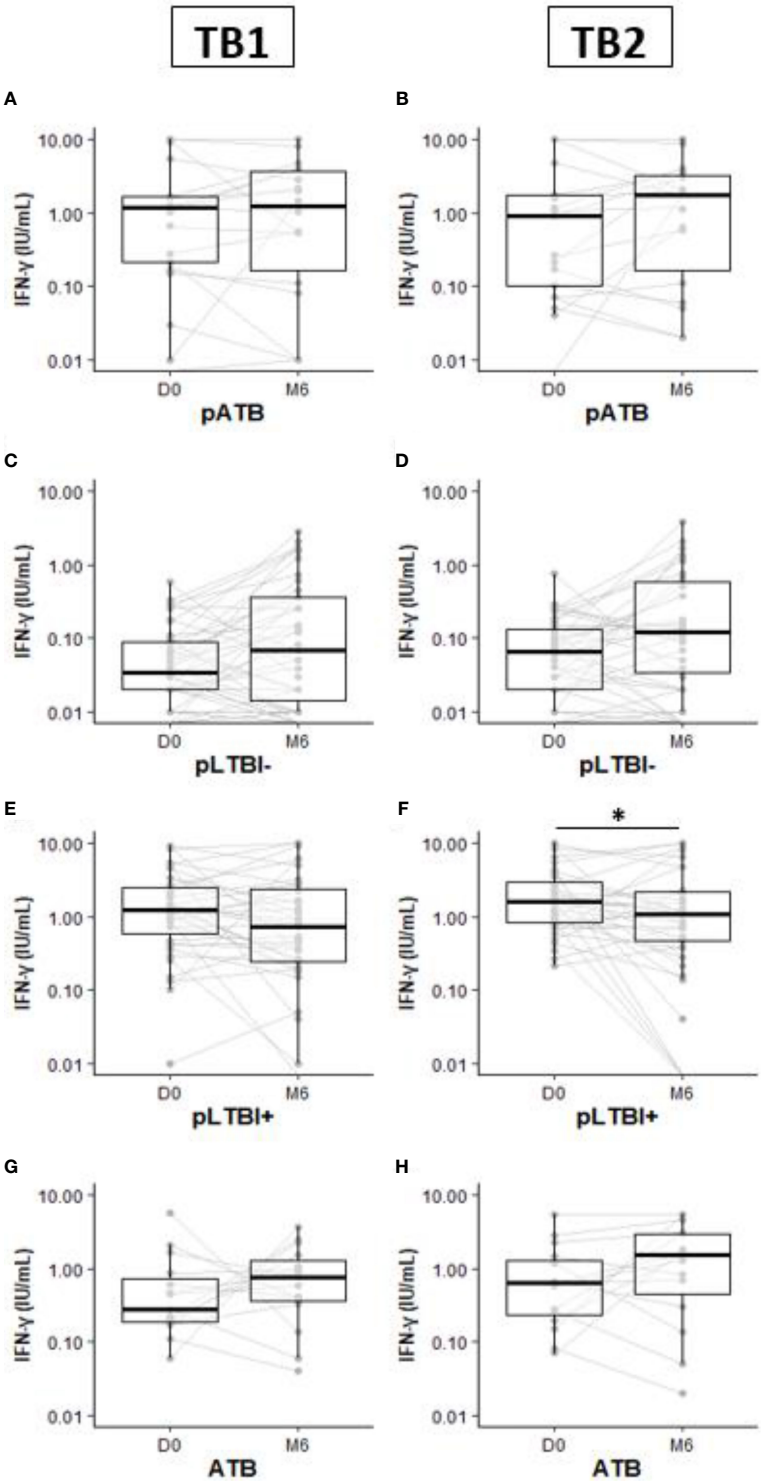

FIGURE 2 | Comparison of IFN-γ production response by QuantiFERON-TB Gold-Plus Assay within pregnant and non-pregnant women subgroups pATB **(A**, **B)**, pLTBI- **(C**, **D)**, pLTBI+ **(E**, **F)**, and ATB **(G**, **H)** between inclusion (D0) and the 6-month follow-up visit (M6). Symbols indicate individual IFN-γ values. Boxes indicate median values. Wilcoxon paired test comparisons were calculated and shown as *$p < 0.05$. DO, inclusion; M6, six months follow up visit; pATB, TB-confirmed pregnant women; ATB, TB-confirmed non-pregnant women; pLTBI-, pregnant women with QFT-P negative response; pLTBI+, pregnant women with QFT P positive response.

TABLE 3 | QuantiFERON-TB Gold-Plus assay results regarding the study participants clinical group.

	Total	QFT-P n (%)		
		Pos	Neg	Ind
HC	35	16 (45.7)	18 (51.4)	1 (2.8)
pNTB	209	72 (34.4)	132 (93.2)	5 (2.4)
pATB	24	14 (58.3)	10 (41.7)	0 (0.0)
ATB	16	10 (62.5)	5 (31.2)	1 (6.2)

QFT-P, QuantiFERON TB Gold Plus; pNTB, non-TB pregnant women; pATB, TB-confirmed pregnant women; ATB, non-pregnant TB-confirmed women; HC, healthy non-pregnant women; Neg, negative; Pos, positive; Ind, indeterminate.

M. tuberculosis-specific antigens in pregnant women independently to their clinical TB groups when compared to their non-pregnant women counterpart with similar childbearing ages. This alteration of the Th1 response in pregnant women is involved in physiological processes known to preserve the fetus (4). Here the present study showed that the concentrations of IFN-γ produced are lower in pregnant women exposed to *M. tuberculosis* (latent infection and active TB) compared to those non-pregnant with active TB (ATB) and healthy controls (HC) baseline concentrations. Moreover, in the non-pregnant asymptomatic women with negative QFT-P responses (LTBI-), this IFN-γ production is higher than in pregnant women having TB symptoms.

After being exposed to *M. tuberculosis*, the host immune system establishes the innate immune response followed by a strong implication of the cell-mediated response including the production of IFN-γ that can activate further cellular responses but can also trigger an intracellular antibacterial role (15). It has been reported that pregnancy impaired the immune response against TB by suppressing the Th1 pro-inflammatory response (16). Hormones associated with pregnancy such as progesterone were suggested to cause the placenta to produce cytokines such as IL-10 that suppress the production of Th1 cytokines, including IFN-γ (17, 18). Such changes lead then to an altered immune system status and could impair the capacity to produce TB-specific IFN-γ (19). While our study seems to confirm this global decline in the IFN-γ responses observed from other longitudinal studies in pregnant women (7, 20), the additional parallel comparison of the IFN-γ response with that of control non-pregnant women of similar age in our study showed that this IFN-γ response is strongly requested following an exposition to *M. tuberculosis* in pregnant women. Similar immune responses with a strong IFN-γ production were observed among LTBI+ pregnant women in India after the comparison with LTBI- pregnant women (10). This strong activation of IFN-γ following an exposition to *M. tuberculosis* and observations made on the antiviral immune response in pregnant woman seems to confirm the fact that pregnancy is not a period of immunosuppression notably for Th1 but a temporary attenuation in immune responses characterized by a strengthening of innate immune barriers and a concomitant reduction in pro-inflammatory immunity that is resumed when exposed to infections (21, 22).

After delivery, the IFN-γ magnitude seems to return to its baseline concentration except for the former active TB group that was successfully treated in which the *M. tuberculosis*-specific IFN-γ response remains high and positive regarding the QFT-P response. All the previous active TB pregnant women (pATB) remained indeed positive after 6 months of follow-up with the QFT-P assay. This phenomenon could be explained by the postpartum immune reconstitution syndrome (23). Physiologic or enhanced proinflammatory response (especially Th1) during the postpartum period and reversal in the cytokine pattern 3–6 weeks after delivery were also previously observed (24). However, as the study was conducted in a high TB incidence

TABLE 4 | QuantiFERON TB Gold-Plus (QFT-P) assay results stratified by characteristics of the pregnant women group at the inclusion (D0).

Age	Total	QFT-P D0		*p value*
		Pos	Neg	
<20	45	13 (28.8)	32 (71.1)	
20–24	49	20 (40.8)	29 (59.1)	0.2
25–29	69	21 (30.4)	48 (68.6)	1
≥30	65	31 (47.7)	34 (52.3)	0.04
TB contact history				
Yes	52	28 (53.8)	24 (46.1)	0.0037
No	176	55 (31.2)	121 (68.8)	
TB treatment history				
Yes	20	14 (70.0)	6 (30.0)	0.0036
No	208	69 (33.1)	139 (66.8)	
Birth course				
Gave birth	125	55 (44.0)	70 (56.0)	0.48
Ongoing pregnancy	22	12 (54.5)	10 (45.5)	

TB, tuberculosis; QFT-P, QuantiFERON TB Gold Plus; Neg, negative; Pos, positive.

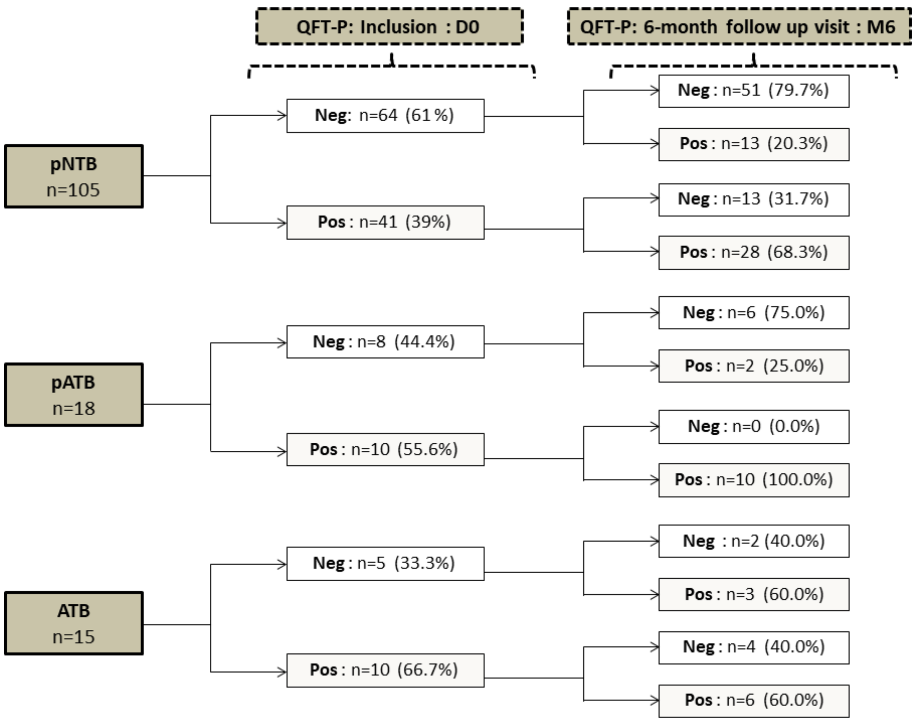

FIGURE 3 | Qualitative QuantiFERON-TB Gold-Plus Assay conversion and reversion proportion within pregnant women (pNTB and pATB) and TB-confirmed non-pregnant women (ATB) groups between inclusion (D0) and the 6-month follow-up visit (M6). QFT-P, QuantiFERON-TB Gold Plus; Neg, negative; Pos, positive; DO, inclusion; M6, six months follow up visit; pNTB, non-TB pregnant women; pATB, TB-confirmed pregnant women; ATB, TB-confirmed non-pregnant women.

area, a reinfection or a continuous exposition to TB has to be considered especially in those that had the TB symptoms.

Finding TB-related symptoms in pregnant women that are immunoreactive to *M. tuberculosis* antigen but without any bacteriological confirmation (pLTBI+) may indicate an immune control of the subclinical form of the disease that is limiting the active replication of the bacteria (25). On the other hand, the observed decline in IFN-γ after 6 months of follow-up in those non-confirmed TB pregnant women with positive QFT-P may explain the risk of postpartum TB already observed in populations from high-incidence areas as is the case in Madagascar for this study (8, 26, 27). However, according to our observations, despite a global decrease in the IFN-γ response during pregnancy, a sufficiently protective IFN-γ response seems to be protective and allows to contain an infection because even after a 6-month follow-up, no women with positive QFT-P did develop any active TB disease form.

Lastly, due to the pregnancy-associated immunologic and physiologic changes that had been reported to have influence on the performance of IGRA (28, 29), the choice of a threshold concentration for definition of a positive reaction for QFT-P has been debated, especially since results close to the recommended cutoff concentration (0.35 IU/ml) can be subject to variability. In the current study, we assumed the use of the recommended cutoff concentration of 0.35 IU/ml rather than a lower threshold concentration as suggested by other researchers (30) to allow the assessment of longitudinal variations and comparison of the IFN-γ concentrations with non-pregnant control that would be difficult with specific cutoff concentration per clinical group.

Our study has several limitations. In the present study, only four pregnant women in the first trimester of pregnancy were enrolled and the majority of the participants in study were recruited at their second and third trimesters of pregnancies. This did not allow further robust statistical comparisons of IFN-γ concentrations regarding gestational age. The gradually occurring changes in cytokine production including IFN-γ responses over the course of the pregnancy have been reported to be more pronounced in the second and third trimesters (8, 21). Second, we have not investigated other TB-specific cytokines. There are changes among other cytokines that are specific to pregnancy (10). The next steps for this study would be to better understand the impact of these variations by assessing other cytokines and biomarkers of LTBI and/or progression to active TB. Third, due to the sample size and the limited data collected from the participants, we did not investigate the association between the increase in IFN-γ concentration and the pregnancy outcomes (delivery mode, birth term, infant outcomes) until the 6-month follow-up visits. However, in this study we found that the proportion of caesarean delivery was higher in those pregnant women with an elevated IFN-γ response independently of the TB clinical issue (data not shown).

To conclude, this study gives an overview of the immune response dynamics during pregnancy and postpartum in *M.*

tuberculosis-infected women. Our findings suggested that despite the decline in IFN-γ response observed during pregnancy, a high concentration of *M. tuberculosis*-induced IFN-γ production was observed in pregnant women with a controlled infection and a lower concentration of this cytokine is observed in those with active TB that however triggers persistent positive QFT-P after the TB treatment. Early detection of *M. tuberculosis* infection followed by preventive treatment administration was suggested to be critical to the global TB control and eradication efforts. More attention should be made with pregnant women as they had a specific immune status and could be vulnerable to *M. tuberculosis* infection and reactivation. These findings suggest a need for implementation of improved strategies to scale up *M. tuberculosis* infection screening among high-risk pregnant women to advance in TB elimination and/or impact the TB-related morbidity and mortality especially in those high-risk pregnant women and their infants in high burden settings.

DATA AVAILABILITY STATEMENT

The raw data supporting the conclusions of this article will be made available by the authors on request to the corresponding author.

ETHICS STATEMENT

The studies involving human participants were reviewed and approved by Comité d'éthique pour la recherche Biomédicale of the Ministry of Public Health of Madagascar. Written informed consent to participate in this study was provided by the participants' legal guardian/next of kin.

AUTHOR CONTRIBUTIONS

NR and PR conceived the idea of this article and provided the framework. All authors collected and analyzed the relevant information. PR undertook the statistical analysis. PR wrote the first draft of the manuscript. The initial draft was revised by all authors. All authors read and approved the final manuscript. All authors contributed to the article and approved the submitted version.

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The Kynurenine/Tryptophan Ratio Is a Sensitive Biomarker for the Diagnosis of Pediatric Tuberculosis Among Indian Children

Jeffrey A. Tornheim[1,2], Mandar Paradkar[3], Henry Zhao[4], Vandana Kulkarni[3], Neeta Pradhan[3], Aarti Kinikar[5], Anju Kagal[5], Nikhil Gupte[2,3], Vidya Mave[2,3], Amita Gupta[1,2,6] and Petros C. Karakousis[1,2,6] for the CTRIUMPh RePORT India Study Team*

[1] Center for Tuberculosis Research, Department of Medicine, Johns Hopkins University School of Medicine, Baltimore, MD, United States, [2] Center for Clinical Global Health Education, Department of Medicine, Johns Hopkins University School of Medicine, Baltimore, MD, United States, [3] Byramjee Jeejeebhoy Government Medical College-Johns Hopkins University Clinical Research Site, Pune, India, [4] Johns Hopkins University, Baltimore, MD, United States, [5] Byramjee Jeejeebhoy Government Medical College, Pune, India, [6] Department of International Health, Johns Hopkins Bloomberg School of Public Health, Baltimore, MD, United States

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***Correspondence:**
Jeffrey A. Tornheim
tornheim@jhu.edu

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Objectives: Pediatric tuberculosis (TB) remains difficult to diagnose. The plasma kynurenine to tryptophan ratio (K/T ratio) is a potential biomarker for TB diagnosis and treatment response but has not been assessed in children.

Methods: We performed a targeted diagnostic accuracy analysis of four biomarkers: kynurenine abundance, tryptophan abundance, the K/T ratio, and IDO-1 gene expression. Data were obtained from transcriptome and metabolome profiling of children with confirmed tuberculosis and age- and sex-matched uninfected household contacts of pulmonary tuberculosis patients. Each biomarker was assessed as a baseline diagnostic and in response to successful TB treatment.

Results: Despite non-significant between-group differences in unbiased analysis, the K/T ratio achieved an area under the receiver operator characteristic curve (AUC) of 0.667 and 81.5% sensitivity for TB diagnosis. Kynurenine, tryptophan, and IDO-1 demonstrated diagnostic AUCs of 0.667, 0.602, and 0.463, respectively. None of these biomarkers demonstrated high AUCs for treatment response. The AUC of the K/T ratio was lower than biomarkers identified in unbiased analysis, but improved sensitivity over existing commercial assays for pediatric TB diagnosis.

Conclusions: Plasma kynurenine and the K/T ratio may be useful biomarkers for pediatric TB. Ongoing studies in geographically diverse populations will determine optimal use of these biomarkers worldwide.

Keywords: biomarker, transcriptomics, metabolomics (OMICS), diagnostics, pediatric tuberculosis

INTRODUCTION

Tuberculosis (TB) is a leading global cause of morbidity and mortality, and is likely to re-emerge as a primary cause of death from infection following the current global pandemic (1). Diagnosis in young children, however, remains a significant challenge due to their inability to produce adequate sputum samples, the frequency of extrapulmonary disease, and the overall paucibacillary nature of pediatric infections (2). In that context, many studies have sought to identify host-derived markers of infection in children that do not rely on direct detection in clinical samples of *Mycobacterium tuberculosis (*Mtb), the bacterium that causes TB (3–5). A recent series of studies highlights the increased kynurenine concentration and lower tryptophan concentration in the blood of patients with pulmonary TB (6, 7). The resulting ratio of kynurenine to tryptophan (K/T) has therefore been proposed as a potential biomarker for the diagnosis of TB that was also associated with treatment outcomes. The decline in tryptophan is associated with the induction of immunoregulatory enzyme indoleamine 2,3-dioxygenase (IDO-1), which breaks down tryptophan to kynurenine metabolites and suppresses the immune response, particularly through shifts in macrophage metabolism and induction of T-cell anergy and apoptosis (8, 9). Metabolic changes in this pathway have also been identified among those with latent TB infection, as well as in multidrug-resistant TB, cavitary disease, and extrapulmonary TB, with low tryptophan in the cerebrospinal fluid of people with tuberculous meningitis (7, 10). Moreover, changes in tryptophan catabolism returned to normal during TB treatment, suggesting a role as a marker of TB disease activity and treatment response. The K/T ratio and IDO-1 activity have been associated with diagnosis of TB and response to treatment in other special populations of interest, such as people with HIV (11), but not yet in children. In order to assess the roles of kynurenine, tryptophan, the K/T ratio, and IDO-1 gene expression as diagnostic biomarkers and indicators of successful treatment response, we performed a secondary analysis of data from two previous studies of pediatric transcriptomic and metabolomic profiling (3, 4).

MATERIALS AND METHODS

The data were initially collected as part of a nested laboratory substudy from a 5-year prospective observational cohort of adults and children with tuberculosis (cases) and the household contacts of participants with pulmonary tuberculosis (controls) described in previous publications (12). Study participants were enrolled at the Byramjee Jeejeebhoy Government Medical College ("BJGMC"), a tertiary teaching hospital in Pune, India in collaboration with researchers from Johns Hopkins University. Samples were collected from study participants at the time of enrollment, as well as longitudinally throughout treatment for cases and for 1 year after enrolment for controls. Study participants under 15 years of age from Pune, India with confirmed tuberculosis had PAXgene tubes selected from the study biorepository for unbiased transcriptomic analysis (3) and plasma samples selected for integrated unbiased metabolomic analysis (4). Participants had tuberculosis confirmed by a combination Xpert MTB/RIF (6 participants), culture (6 participants), or the presence of granulomas on histopathology of extrapulmonary specimens (7 participants, not mutually exclusive). All cases were successfully treated to cure with a combination of isoniazid, rifampin, pyrazinamide, and ethambutol, and each case was age and sex-matched with 2 controls for these analyses. All cases and controls were HIV negative and all controls were ruled out for active tuberculosis at the time of enrolment by symptom screen and chest X-ray, and rule out for latent tuberculosis infection (LTBI) by tuberculin skin test (TST) and interferon gamma release assay (IGRA) at the time of enrolment. Whole blood samples were collected from cases for transcriptional profiling at baseline, 1 month, and 6 months of treatment, and from controls at enrolment, month 4-6, and month 12 after enrolment. Plasma samples were collected on the same schedule for metabolomic profiling. TST and IGRA testing were repeated at each visit to identify new latent or active tuberculosis over the first 12 months after enrolment. No controls developed active TB during that period.

Transcriptional profiling was performed after RNA isolation from PAXgene tubes and sequenced by Illumina HiSeq 2500, aligned to the human genome (GRCh38.10) using the STAR aligner and annotated using GENCODE (13). Differential expression analysis was conducted in R using DESeq2. This analysis identified a 71-gene diagnostic signature and a 25-gene treatment response signature for pediatric tuberculosis. Metabolomic profiling was performed with the automated MicroLab STAR system using Waters ACQUITY ultra-performance liquid chromatography (UPLC), a Thermo Scientific Q-Exactive high resolution/accurate mass spectrometer and Orbitrap mass analyzer operated at 35,000 mass resolution with a scan range of 70-1000m/z (14, 15). Peaks were identified using a standardized commercially available library of known compounds (16). Differentially expressed metabolites were identified between groups and a random forest decision tree identified the simplest combinations of metabolites that differentiated groups with the greatest accuracy. Integrated multi-omics analysis identified the relative contributions of metabolomic and transcriptomic data to the optimal features that diagnosed tuberculosis and identified treatment response in children. To control for multiple comparisons, differential expression and abundance was defined as ≥2-fold difference between groups with a Benjamini-Hochberg false discovery rate of <0.05 applied to correct for multiple comparisons. Data from those studies are available from NCBI (accession code PRJNA588242) and in the supplementary files of our prior publications (4).

In the present analysis, we combine the two published datasets to determine the extent to which the tryptophan, kynurenine, the K/T ratio, and IDO-1 were able to discriminate groups of children with and without TB (i.e., as a diagnostic biomarker), and children with TB over time from the start of treatment, after 1 month of treatment, and at the end of 6 months of successful treatment (i.e., as a biomarker of treatment

response). Relative abundance of metabolites and gene expression levels were correlated between participants and summarized by R^2 levels. Relative abundance between study groups and time points was assessed by Wilcoxon tests for paired samples and the Kruskal-Wallis test for multiple groups, with differences of $p<0.05$ considered significant. Receiver operator characteristic (ROC) curves were calculated for each potential biomarker using the pROC package in R with optimal thresholds determined by Youden's index and overall accuracy presented by area under the ROC curve (AUC) [25].

RESULTS

Participants

Participants with TB had a median age of 8.5 years (interquartile range, IQR=6.8-12), half were male, and half had only pulmonary tuberculosis only. All cases and controls were HIV seronegative. No controls had active TB or latent TB infection at the time of enrollment, and none developed active TB in the subsequent year, but 13 (40.6%) developed positive TST or IGRA during the study.

Correlation Between Biomarkers and Unbiased Analysis

Tryptophan and kynurenine abundance were poorly correlated with contemporaneous IDO-1 transcript abundance across all study time points ($R^2<0.001$ and $R^2 = 0.014$, respectively), with similar poor correlation between IDO-1 and the K/T ratio ($R^2 = 0.023$) and better correlation between tryptophan and kynurenine levels ($R^2 = 0.347$). Kynurenine demonstrated a small but significant decrease among cases between the start and end of treatment (-0.029-fold, p=0.037), although this effect was not apparent until the completion of treatment (**Table 1** and **Figure 1**). Neither kynurenine or tryptophan abundance, nor IDO-1 expression was significantly different between any of the other groups compared for either diagnosis, treatment response, or incident tuberculosis infection among controls during the study period.

Biased Assessment for Diagnosis and Treatment Response

Next, we assessed each biomarker individually by ROC analysis and without controlling for multiple comparisons (as was performed in the unbiased analysis above). None of the biomarkers significantly differentiated cases from controls, though kynurenine abundance was higher among cases than controls (p=0.051). Despite non-significant differences between groups in unbiased analysis, kynurenine and the K/T ratio achieved good discrimination of cases from controls (AUC 0.667 and AUC 0.676 for kynurenine and the K/T ratio, respectively). These biomarkers performed less well as measures of treatment response, with AUCs of 0.494-0.606 (**Table 2**). Subgroup analysis did not find that discrimination ability improved when comparisons were limited to only controls without incident TB during follow-up. None of these biomarkers demonstrated consistent changes in abundance or expression over time by study group that would suggest use as an indicator of clinical improvement during treatment or as an early marker of incident latent infection in children (**Figure 1**).

DISCUSSION

As the world recovers from the COVID-19 pandemic, it is likely that we will see a rise in reported TB cases in the coming years. Improved tools for the diagnosis of pediatric TB and non-sputum based markers of treatment response are needed to reduce the morbidity and mortality of these new cases. When new host biomarkers of tuberculosis are identified, it is imperative that these be assessed in special populations, including children, to confirm if and how they can be best employed. In this secondary analysis of metabolomic and transcriptomic data, we evaluated the performance of the following four biomarkers for the diagnosis of pediatric TB and assessment of response to successful treatment: kynurenine, tryptophan, the K/T ratio, and IDO-1 gene expression. We found that plasma kynurenine levels declined over the course of TB treatment, and that both plasma kynurenine and the plasma K/T ratio achieved AUCs >0.66, with the K/T ratio achieving a sensitivity of 82% and a specificity of 56% for diagnosis of pediatric TB in this population. The other biomarkers assessed did not perform as well for either diagnosis or response to treatment. This suggests that the K/T ratio has acceptable accuracy for use as a diagnostic tool in children with TB, meeting the WHO target product profile by achieving better sensitivity than existing commercial diagnostic tools for pediatric TB (17).

TABLE 1 | Differential abundance and expression of kynurenine, tryptophan, and IDO-1 between study groups.

	Kynurenine Abundance		Tryptophan Abundance		IDO-1 Expression	
Groups Compared	**Fold Change**	**Log_{10} p-value**	**Fold Change**	**Log_{10} p-value**	**Fold Change**	**Adjusted p-value**
Cases vs. Controls	0.345	1.046	0.090	0.212	0.320	0.668
Cases Starting Treatment vs. Treatment Month 1	-0.263	0.600	0.120	0.283	0.273	0.585
Cases at Treatment Month 1 vs. End of Treatment	0.234	0.415	-0.044	0.101	-0.198	0.779
Cases Starting vs. Finishing Treatment	-0.029	0.037	0.077	0.181	-0.090	0.803[1]
Cases at the End of Treatment vs. New LTBI	0.473	1.097	0.094	0.177	2.362	0.769
Controls with New LTBI vs. Controls without New LTBI	-0.171	0.432	-0.138	0.250	0.902	0.531

[1]*Adjusted p-value could not be calculated, unadjusted p-value presented.*

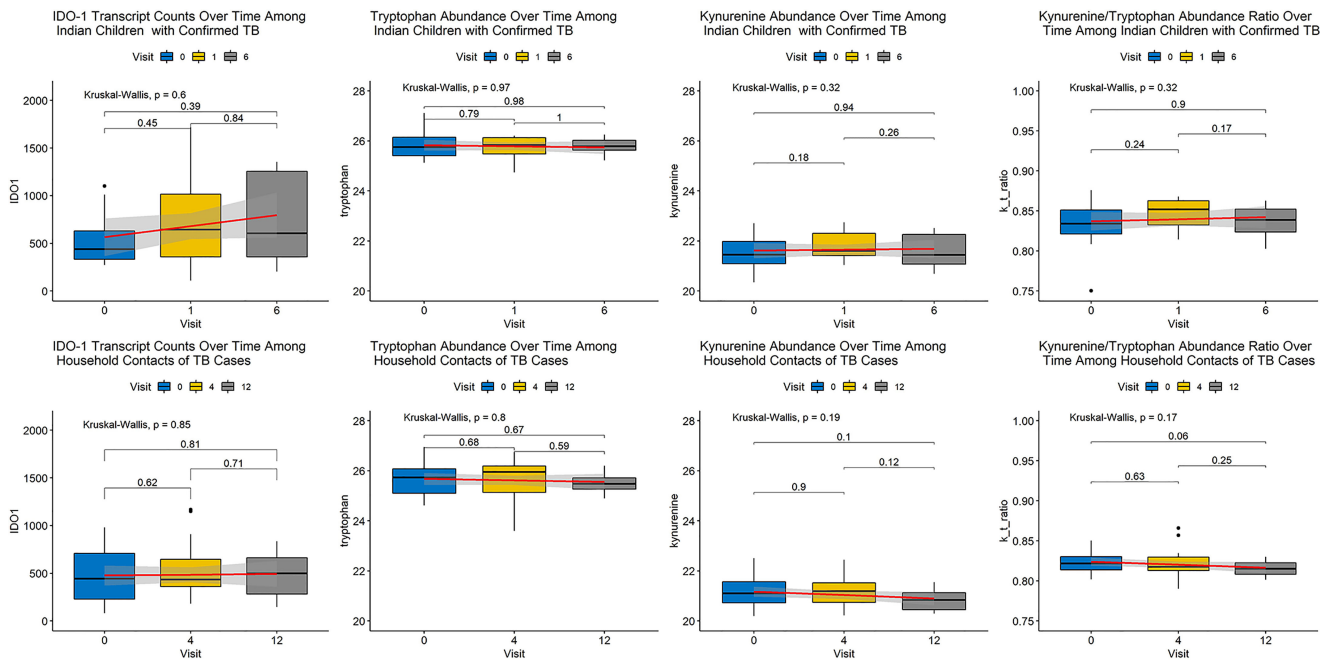

FIGURE 1 | Changes in Metabolite Abundance and Transcript Expression Over Time Among Cases and Controls. Abundance of each biomarker of interest is expressed as a boxplot with a smoothed regression line in red indicating change over time at each visit, indicated by month of visit. Shading around each red line indicates 95% confidence around the trend. The top row of panels contains plots for cases and the bottom row of panels indicates plots for controls. Each column represents a distinct biomarker with IDO-1 transcript expression in the left column, followed by tryptophan abundance, kynurenine abundance, and the kynurenine/tryptophan (K/T) ratio moving from left to right. None of these biomarkers demonstrated a significant change over duration of follow-up among cases or controls.

IDO-1 activity is a compelling target biomarker for TB diagnostics due to multiple studies demonstrating changes in this pathway in a variety of types of TB, as well as the availability of an inhibitor, 1-methyl-tryptophan, that has been associated with clinical improvement and increased *Mtb* killing (18). The absence of correlation between contemporary IDO-1 transcript abundance and tryptophan and kynurenine levels in this study suggest that post-transcriptional activity is important to this relationship. It is worth noting, however, that while the K/T ratio had reasonable test characteristics for TB in our data, it did not perform as well as other biomarkers we have assessed. Our previous analysis found that a combination of other metabolites achieved AUCs of 0.88 for diagnosis of tuberculosis and 0.86 for response to treatment. A single metabolite, N-acetylneuraminate, was found to have an AUC of 0.66 for TB diagnosis, and another, pyridoxate, was found to have an AUC of 0.87 for response to treatment (4). Similarly, a transcriptional profile derived from this dataset achieved higher sensitivity when applied to other published datasets for both diagnosis (up to 85% sensitive) and treatment response (up to 86% sensitive) (3). Likewise, other groups have published transcriptional signatures of pediatric TB with higher AUCs than that achieved by the K/T ratio in these data for diagnosis and treatment response (AUCs of 0.76 for diagnosis and 0.77 for treatment response) (19, 20). Those analyses did not identify blood tryptophan abundance or IDO-1 expression as significant.

In any study of host-derived genetic or metabolomic biomarkers, it is possible that clinical or genetic differences between study populations could impact study results. This analysis only included data from Indian children without HIV, which may limit generalizability to other populations, including those with HIV. Similarly, this analysis did not analyze samples from people with other respiratory infections and will need to be tested in that setting before it could be used for the clinical test of

TABLE 2 | Test characteristics of kynurenine and tryptophan abundance, the K/T ratio, and IDO-1 expression for the diagnosis of pediatric tuberculosis and treatment response.

	Cases vs. Controls				Response to Treatment			
Groups Compared	**AUC**	**Best Threshold**	**Sensitivity**	**Specificity**	**AUC**	**Best Threshold**	**Sensitivity**	**Specificity**
Kynurenine Abundance	0.667	20.955	0.444	0.875	0.513	21.099	0.400	0.750
Tryptophan Abundance	0.602	25.329	0.407	0.938	0.494	25.550	0.800	0.375
K/T Ratio	0.676	0.833	0.815	0.563	0.519	0.842	0.500	0.688
IDO-1 Expression	0.463	648.5	0.333	0.750	0.606	1167	0.400	1.000

TB in children. Previous studies have found the K/T ratio to be useful among people both with and without HIV, independent of HIV treatment initiation (11, 21), as well as among pregnant woman (22), people with diabetes (23), and among diverse populations in East Asia, Sub-Saharan Africa, and Eastern Europe (6, 7, 10, 11). Metabolomic testing is also currently limited by the need for highly specialized, resource-intensive sensitive testing, but like many other tools, could potentially be translated to simpler methods for near-care or point-of-care testing in the future.

Our findings suggest that plasma kynurenine and the K/T ratio may be useful biomarkers for pediatric TB. Additional studies are needed to validate these biomarkers in diverse pediatric populations and in settings with lower TB incidence. Ongoing studies in geographically diverse populations will help determine the optimal use of these biomarkers worldwide.

DATA AVAILABILITY STATEMENT

The data sets presented in this study can be found in online repositories and in the supplemental files of (4). The names of the repository/repositories and accession number(s) can be found below: https://www.ncbi.nlm.nih.gov/, PRJNA588242.

ETHICS STATEMENT

This study was approved by the institutional review boards of Byramjee Jeejeebhoy Government Medical College, the National Institute of Research in Tuberculosis, and the Johns Hopkins University School of Medicine. Written informed consent for participation was provided by the legal guardians of all pediatric participants under the age of consent, and written informed assent was provided by all pediatric participants ≥ 8 years of age.

AUTHOR CONTRIBUTIONS

JT contributed to the study design, sample processing, data analysis, and manuscript preparation. MP contributed to the study design, data collection, data analysis, and manuscript preparation. HZ contributed to manuscript preparation. VK and NP contributed to study design, sample processing, data analysis, and manuscript preparation. AaK and AnK contributed to data collection and manuscript preparation. NG contributed to data analysis and manuscript preparation. VM supervised study activities and contributed to study design, data collection, and manuscript preparation. AG and PK supervised study design, data collection, analysis, and manuscript preparation. All authors contributed to the article and approved the submitted version.

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Immuno-Diagnosis of Active Tuberculosis by a Combination of Cytokines/Chemokines Induced by Two Stage-Specific Mycobacterial Antigens: A Pilot Study in a Low TB Incidence Country

Violette Dirix[1], Philippe Collart[2], Anne Van Praet[1], Maya Hites[3], Nicolas Dauby[4,5], Sabine Allard[6], Judith Racapé[7], Mahavir Singh[8], Camille Locht[9], Françoise Mascart[1] and Véronique Corbière[1]*

[1] Laboratory of Vaccinology and Mucosal Immunity, Université Libre de Bruxelles (U.L.B.), Brussels, Belgium, [2] Biostatistiques du Pôle Santé (BIOPS), Université Libre de Bruxelles (U.L.B.), Brussels, Belgium, [3] Clinique des maladies infectieuses et tropicales, Hôpital Erasme, Université Libre de Bruxelles (U.L.B.), Brussels, Belgium, [4] Department of Infectious Diseases, Centre Hospitalier Universitaire Saint-Pierre, Université Libre de Bruxelles (U.L.B.), Brussels, Belgium, [5] Institute for Medical Immunology, Université Libre de Bruxelles (U.L.B.), Brussels, Belgium, [6] Dienst Interne Geneeskunde - Infectiologie, Universitair Ziekenhuis Brussel (UZ Brussel), Vrije Universiteit Brussel (VUB), Brussels, Belgium, [7] Biomedical Research Center, Erasme Hospital, Brussels, Belgium, [8] Lionex Diagnostics and Therapeutics, Braunschweig, Germany, [9] Univ. Lille, CNRS, Inserm, CHU Lille, Institut Pasteur de Lille, U1019 – UMR9017 – CIIL – Center for Infection and Immunity of Lille, Lille, France

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***Correspondence:**
Françoise Mascart
francoise.mascart@erasme.ulb.ac.be

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Active tuberculosis (aTB) remains a major killer from infectious disease, partially due to delayed diagnosis and hence treatment. Classical microbiological methods are slow and lack sensitivity, molecular techniques are costly and often unavailable. Moreover, available immuno-diagnostic tests lack sensitivity and do not differentiate between aTB and latent TB infection (LTBI). Here, we evaluated the performance of the combined measurement of different chemokines/cytokines induced by two different stage-specific mycobacterial antigens, Early-secreted-antigenic target-6 (ESAT-6) and Heparin-binding-haemagglutinin (HBHA), after a short *in vitro* incubation of either peripheral blood mononuclear cells (PBMC) or whole blood (WB). Blood samples were collected from a training cohort comprising 22 aTB patients, 22 LTBI subjects and 17 non-infected controls. The concentrations of 13 cytokines were measured in the supernatants. Random forest analysis identified the best markers to differentiate *M. tuberculosis*-infected from non-infected subjects, and the most appropriate markers to differentiate aTB from LTBI. Logistic regression defined predictive abilities of selected combinations of cytokines, first on the training and then on a validation cohort (17 aTB, 27 LTBI, 25 controls). Combining HBHA- and ESAT-6-induced IFN-γ concentrations produced by PBMC was optimal to differentiate infected from non-infected individuals in the training cohort (100% correct classification), but 2/16 (13%) patients with aTB were misclassified in the validation cohort. ESAT-6-induced-IP-10 combined with HBHA-induced-IFN-γ

concentrations was selected to differentiate aTB from LTBI, and correctly classified 82%/77% of infected subjects as aTB or LTBI in the training/validation cohorts, respectively. Results obtained on WB also selected ESAT-6- and HBHA-induced IFN-γ concentrations to provided discrimination between infected and non-infected subjects (89%/90% correct classification in the training/validation cohorts). Further identification of aTB patients among infected subjects was best achieved by combining ESAT-6-induced IP-10 with HBHA-induced IL-2 and GM-CSF. Among infected subjects, 90%/93% of the aTB patients were correctly identified in the training/validation cohorts. We therefore propose a two steps strategy performed on 1 mL WB for a rapid identification of patients with aTB. After elimination of most non-infected subjects by combining ESAT-6 and HBHA-induced IFN-γ, the combination of IP-10, IL-2 and GM-CSF released by either ESAT-6 or HBHA correctly identifies most patients with aTB.

Keywords: active tuberculosis, ESAT-6, HBHA, IFN-γ, IL-2, GM-CSF, IP-10

INTRODUCTION

Tuberculosis (TB) remains a leading cause of death in the world, responsible for high morbidity and mortality worldwide with about 10 million new cases in 2020 and 1.5 million deaths (1). Diagnosis and hence appropriate treatment are often delayed due to the wide spectrum of clinical manifestations of active TB (aTB) and to the non-availability of sensitive and specific tests providing a rapid and accurate diagnosis (2, 3). In addition to classical clinical manifestations of aTB, this disease may occur as subclinical TB without suggestive symptoms, or as extrapulmonary TB often pauci-symptomatic in immunocompromised individuals, so that diagnosis strictly based on clinical signs or symptoms is illusive (2). The classical diagnostic method remains the identification of *M. tuberculosis* by direct smear microscopy or by culture that are both low in sensitivity and/or slow. Early diagnosis of infectious cases by sputum microscopy is only possible in approximately 50% of cases. Moreover, people with subclinical TB may likely be missed if TB culture is not performed and this is often the case in asymptomatic individuals. Molecular techniques such as GeneXpert are more sensitive but they are costly and often unavailable in primary-care settings.

Immuno-diagnosis was therefore identified as a promising approach for diagnosis of aTB. However, the commercially available tests, the interferon-γ-release assays (IGRA), based on the release of IFN-γ by blood cells in response to their *in vitro* stimulation with mycobacterial peptides corresponding to antigens encoded in the genomic region of difference (RD)-1 (the early-secreted-antigenic-target-6 (ESAT-6), and the culture-filtrate-protein-10 (CFP-10)), have a relatively high false negative rate in patients with aTB (4–6). In addition, these IGRAs, initially developed to diagnose latent TB infection (LTBI), are positive both in LTBI and in patients with aTB (7). The differential diagnosis of LTBI and aTB is therefore not possible with commercial IGRAs. New generation IGRAs were developped to improve the diagnosis of aTB. They are based either on recent studies indicating that the *M. tuberculosis* specific $CD8^+$ T cell responses are positively correlated with the bacterial load and recent exposure to *M. tuberculosis* for the QuantiFERON-TB Gold Plus, or by inclusion of L-alanine dehydrogenase as an additional antigen for the LIOFeron TB/LTBI (8, 9). The added value of these improvements for the diagnosis of aTB and its differential diagnosis with LTBI was however not confirmed until now (8).

Therefore, several studies aimed to identify other proteins from *M. tuberculosis* as potential candidates to distinguish aTB from LTBI and they often also extend the cytokine measurements beyond IFN-γ to improve differential diagnosis. These studies generally first identify markers of *M. tuberculosis* infection, before applying one or several biomarkers to differentiate aTB from LTBI. Among the numerous proteins evaluated, the mycobacterial heparin-binding haemagglutinin (HBHA, Rv0475) appears as one of the most promising antigens to differentiate LTBI from aTB, but only few studies assessed HBHA-induced cytokines other than IFN-γ (10–12). In contrast, the potential added value of various cytokines induced by the peptides of the commercial IGRAs has been investigated, but with conflicting outcomes, as highlighted by a recent meta-analysis (13).

We previously reported on a combined HBHA- and ESAT-6-IGRA performed on either peripheral blood mononuclear cells (PBMC) or whole blood (WB) to diagnose LTBI and to partially differentiate LTBI from aTB (14–16). Even though this combined IGRA provided a better discrimination between LTBI and aTB than any other available *in vitro* test, it remained imperfect. In this study, we therefore measured cytokines/chemokines other than IFN-γ released in response to HBHA and to ESAT-6 and evaluated the potential added value of a combined analysis of cytokine/chemokine secretion for the differential diagnosis and especially for the diagnosis of aTB.

MATERIAL AND METHODS

Ethics Statement

The study protocol P2011/113 was approved by the ethics committee of ULB-Hôpital Erasme, Brussels, Belgium, and informed written consent was obtained from all participants.

Study Protocol

A panel of 13 different chemokines and cytokines induced by two different mycobacterial antigens after a short-term *in vitro* stimulation of blood cells was measured. Results obtained by *in vitro* stimulation of PBMC were analyzed in parallel with those obtained by stimulating diluted WB. The two antigens, HBHA and ESAT-6, were selected as they are secreted at different stages of the mycobacterial metabolism, in order to cover the whole spectrum of *M. tuberculosis* infection (17, 18). A training cohort was constituted to select the most promising cytokines or chemokines and their best combination to differentiate infected from non-infected subjects and then to identify patients with aTB among infected individuals. The accuracy of the selected combinations was further evaluated on samples from an independent validation cohort.

The Training and the Validation Cohorts

To ensure the reproducibility of the results and to limit the influence of a possible inclusion bias, two independent cohorts were evaluated. The training or discovery cohort comprised 61 individuals prospectively enrolled as being potentially *M. tuberculosis* infected (LTBI or aTB) or not (non-infected controls). For most of them (n=51), residual supernatants from stimulated PBMC or WB used in a previous study were used (16). Ten additional individuals (six controls, three LTBI, one aTB) were newly included. Samples from this cohort allowed us to identify the most suitable markers to first differentiate infected from non-infected subjects and then to differentiate aTB from LTBI subjects. These findings were then confirmed in a validation cohort that was independent from the training cohort and comprised 69 subjects included in a previous study (16). Residual supernatants from this cohort were used to confirm the diagnostic performance of the best combinations of markers identified on the training cohort (**Table 1**).

For both cohorts, the individuals were classified in three different groups, non-infected controls, LTBI subjects and patients with aTB, based on classical criteria as reported (19). Briefly, both non-infected controls and LTBI subjects were healthy with a negative or positive (induration size of 10 mm in case of risk factor and of 15 mm for the others) tuberculin skin test (TST) respectively. LTBI subjects also had a chest radiograph with no signs of aTB infection. Active TB diagnosis was based on microbiological proof for most patients, comprising both pulmonary and extrapulmonary aTB (**Table 1**). Based on these criteria, the training cohort comprised 17 non-infected controls, 22 LTBI subjects and 22 aTB patients, whereas the validation cohort comprised 25 non-infected controls, 27 LTBI subjects and 17 aTB patients. The main demographic data of the subjects included in this study for the two cohorts are reported in **Table 1**. Differences between the median ages and the sex ratios of the individuals included in the two cohorts were minor (**Table 1**). The proportion of subjects originating from endemic countries was lower for controls than for infected individuals ($p \leq 0.05$) and was the most elevated among aTB patients (**Table 1**).

Induction of Chemokine and Cytokine Secretions

PBMC and diluted WB were *in vitro* stimulated as reported elsewhere (16, 19). Briefly, 1.10^6 PBMC, suspended in 500 µl culture medium (RPMI with 1 ng/ml IL-7), or 250 µl WB diluted 1:1 in IL-7-enriched-AIMV medium, were incubated during 24hrs at 37°C (5% CO_2) with HBHA purified from *Mycobacterium bovis* BCG as described (20) (1 µg for PBMC and 2 µg for WB) or with recombinant ESAT-6 (2.5 µg) provided by Lionex (Diagnostics & Therapeutics GmbH, Braunschweig, Germany), before supernatant collection. Cells incubated in antigen-free medium and incubated with staphylococcal enterotoxin B (SEB, Sigma-Aldrich, Bornem, Belgium; 0.5 and 1 µg/ml for the PBMC and diluted WB stimulation, respectively) served as negative and positive controls, respectively.

Chemokine and Cytokine Concentration Measurements

The concentrations of 13 chemokines or cytokines were measured by multiparameter-based immunoassays (Milliplex human

TABLE 1 | Demographic and clinical data from the training and validation cohorts.

	aTB patients		LTBI individuals		Non-infected controls	
	Training cohort	Validation cohort	Training cohort	Validation cohort	Training cohort	Validation cohort
N	22	17	22	27	17	25
Median age (range) (yrs)	33 (19-60)	40 (18-63)	31 (21-64)	49° (19-64)	35 (21-60)	43 (21-61)
Male (no. [%])	16* (73)	9** (56)	8 (36)	12 (44)	10 (59)	5°° (20)
Ethnic origin (no. [%])						
Caucasian	8 [36]	3 [18]	13 [59]	19 [59]	16 [94]	24 [96]
North African	6 [27]	7 [41]	3 [14]	6 [19]	1 [6]	1 [4]
Sub-Saharan African	6 [27]	6 [35]	4 [18]	6 [19]	0 [0]	0
Other	2 [9]	1 [6]	2 [9]	1 [3]	0 [0]	0
Clinical data						
Pulmonary TB (no. [%])	14 [64]	12 [71]	NA	NA	NA	NA
Extrapulmonary TB (no. [%])	8 [36]	5 [29]	NA	NA	NA	NA
Positive sputum smear/culture/PCR (no. [%])	19 [86]	16 [94]	NA	NA	NA	NA

N, number; LTBI, Latent Tuberculosis Infection; aTB, active Tuberculosis; NA, not applicable.

*°p=0.0014 vs LTBI subjects from the training cohort; *p=0.0329 versus LTBI subjects from the same cohort; **p=0.0229 versus controls from the same cohort °°p=0.0202 vs CTRL subjects from the training cohort.*

cytokine/chemokine kits-Merck, Belgium) according to the manufacturer's instructions: granulocyte macrophage colony-stimulation factor (GM-CSF), Growth related oncogene (GRO), IFN-γ, interleukin (IL)-1β, IL-2, IL-6, IL-8, IL-10, IL-17A, IFN-γ-induced protein 10 (IP-10), Macrophage inflammatory protein (MIP)-1α, soluble CD40 ligand (sCD40L), and Tumor necrosis factor-alpha (TNF-α). Culture supernatants were diluted using dilution factors specific to each analyte in order to obtain concentrations within an interpretable range. Results were analysed with a Bio-Plex® MAGPIX™ Multiplex reader, Bio-Plex Manager™ MP Software and Bio-Plex Manager 6.1 Software (BIO-RAD laboratories, Nazareth Eke, Belgium). If detectable, the analyte concentrations obtained in the antigen-free conditions were subtracted from those obtained with antigen stimulation. To allow statistical analyses with continuous variables, the concentrations below the detection limit were allocated the arbitrary value of half of the threshold of detection, whilst results exceeding the assay's upper limit of detection were attributed the concentration corresponding to this limit. The laboratory scientist performing the sample analysis was blinded to the clinical and other laboratory data.

Statistical Analysis

Differences in the concentrations of chemokines/cytokines between groups of subjects were analysed using the Mann-Whitney U test. The diagnostic abilities of individual parameters were first assessed by receiver operator characteristics (ROC) curve analysis and the areas under the curve (AUC) were calculated (GraphPad Prism version 7.03, GraphPad Software, La Jolla California USA, www.graphpad.com). The host marker selection was further evaluated by random forest analysis (random Forest package version 4.6-14) and the predictive abilities of combinations of markers were investigated by logistic regression using R (R-4.0.3, R Foundation for Statistical Computing, Vienna, Austria). Results were graphically represented with the linear predictor of the logistic regression. The tests were considered statistically significant when the p-value was < 0.05.

RESULTS

Selection of PBMC-Produced Markers to Identify *M. tuberculosis*-Infected Subjects

We first investigated the ability of an individual marker, or a combination of markers, to differentiate *M. tuberculosis*-infected from non-infected individuals in the training cohort using ESAT-6 and HBHA for *in vitro* stimulation. Cytokine/chemokine concentrations were generally significantly higher for infected than non-infected subjects (**Supplementary Table 1**). The diagnostic accuracy of each individual marker was assessed by ROC curve analyses and most AUCs were higher than 0.7. We then performed random forest analyses including the 13 analytes secreted in response to ESAT-6 and HBHA to select the optimal combination to differentiate infected from non-infected subjects. The best discrimination was obtained by HBHA-induced IFN-γ, GM-CSF and IL-2, combined with ESAT-6-induced IFN-γ and IL-8 (**Supplementary Figure 1A**).

These markers were further evaluated in different combinations by logistic regression analysis for the training cohort. This analysis identified the combination of HBHA- and ESAT-6-induced IFN-γ as optimal to differentiate infected from non-infected subjects with 95% correct classification of the individuals (**Figure 1A**). Three non-infected subjects were misclassified. This combination was further applied on the validation cohort. In this case, 88% of the subjects were correctly classified with four controls and four infected subjects (two LTBI subjects and two patients with aTB) misclassified (**Figures 1B**, **2A**).

Selection of PBMC-Produced Markers to Differentiate aTB From LTBI

A second random forest analysis including the same markers was performed to select the optimal combination to differentiate aTB from LTBI in the training cohort. ESAT-6-induced IP-10, IL-8, and HBHA-induced IP-10, IFN-γ, and TNF-α were selected (**Supplementary Figure 1B**) and combinations of these cytokines/chemokines were evaluated by logistic regression analysis. The most discriminant combination was ESAT-6-induced IP-10 combined with HBHA-induced IFN-γ, which provided a correct classification of 82% of the individuals: three LTBI subjects were classified as aTB, whereas five patients with aTB were classified as LTBI (**Figure 3A**). The clinical characteristics of the five aTB patients who were misclassified are reported in **Table 2** (n°1 to n°5). The accuracy of this combination was further evaluated on the validation cohort. As in the validation cohort, two aTB patients and two LTBI subjects were misclassified by the first combination of markers (see **Figure 1B**), all LTBI and aTB individuals were included in this analysis. Seventy-four percent of the infected subjects were correctly classified with five LTBI subjects classified as aTB and six patients with aTB classified as LTBI (**Figure 3B**). The clinical characteristics of the misclassified aTB are reported in **Table 3** (n°1 to 6). Two of them were already missed with the initial combination aiming to discard non-infected controls (n°1 and 2). The five misclassified LTBI subjects were considered at risk to reactivate the infection based on previously defined criteria (21) (**Figure 3B**, open circle).

A good performance of the differential diagnosis between aTB and LTBI was thus possible in most cases by combining ESAT-6-induced IP-10 to HBHA-induced IFN-γ. However, this combination did not allow us to identify the two aTB patients who were initially misclassified as non-infected controls. The two-steps approach with a first identification of infected subjects, followed by a differential diagnosis between aTB and LTBI among infected individuals remains thus recommended as illustrated in **Figure 2**, panel A and B respectively. This two-steps approach resulted for the two cohorts in a correct identification of 36/38 aTB patients as infected (**Figure 2A**), with 27 of them as aTB patients (**Figure 2B**).

Selection of WB Markers to Identify *M. tuberculosis*-Infected Subjects

The same approach was applied for the WB assays. Similar to the PBMC, cytokine concentrations were generally significantly higher

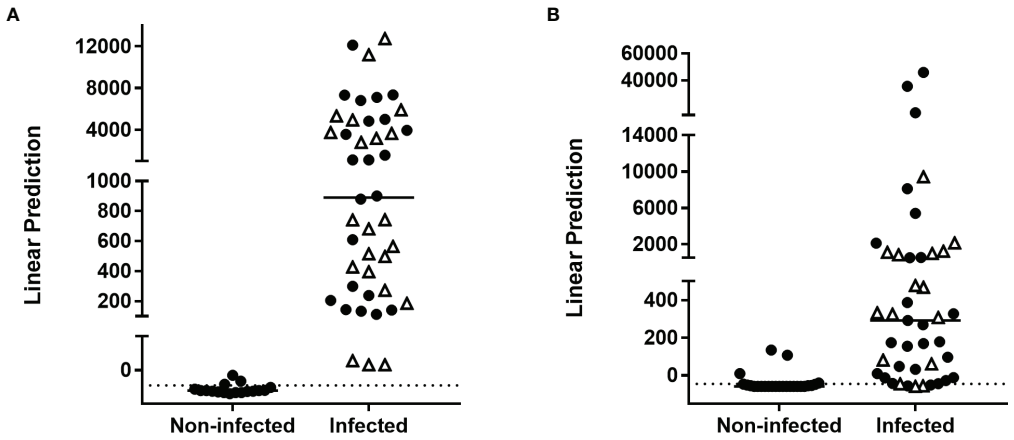

FIGURE 1 | Combination of *M. tuberculosis*-specific immune markers allowing the distinction between *M. tuberculosis*-infected versus non-infected subjects in the PBMC assay. HBHA-IFN-γ and ESAT-6-IFN-γ were the markers selected by logistic regression analysis to be combined for the PBMC-based assay to discriminate *M. tuberculosis*-infected from non-infected subjects. Patients with aTB are indicated by open triangles. Results are represented with their linear predictor for the validation **(A)** and training **(B)** cohorts. The horizontal lines represent the medians and the dotted lines arbitrary cut-offs.

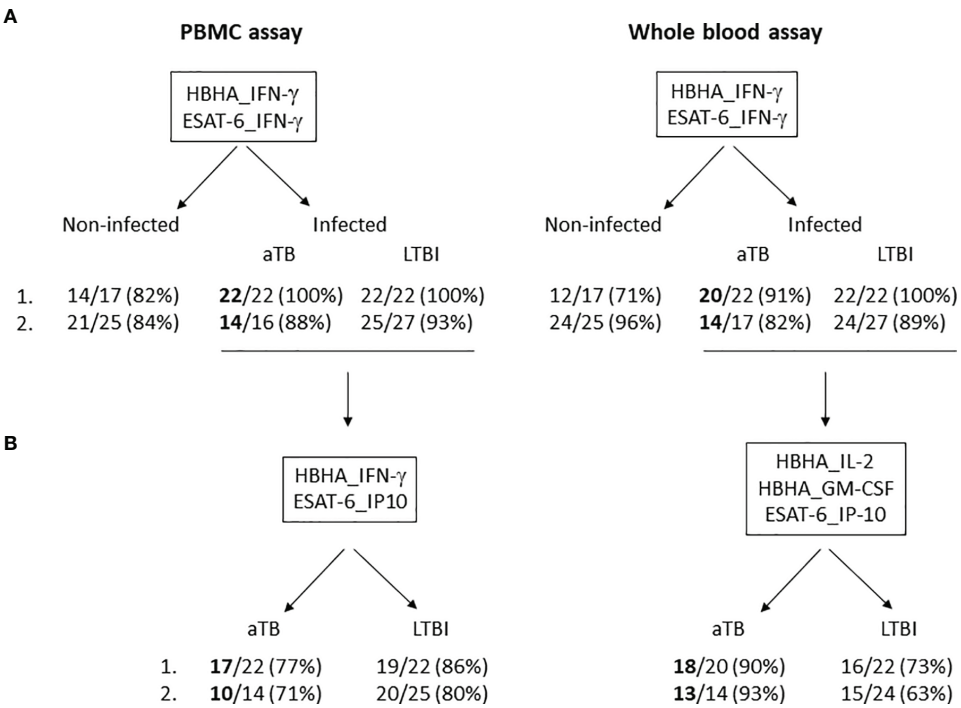

FIGURE 2 | Two-step algorithm for the identification of aTB and LTBI among *M. tuberculosis*-infected subjects by using a combination of cytokines/chemokines induced by two stage-specific mycobacterial antigens in a PBMC- and WB-based assays. **(A)** *M. tuberculosis*-infected subjects, including aTB patients as well as LTBI subjects, were discriminated from non-infected subjects by using a combination of HBHA-IFN-γ and ESAT-6-IFN-γ in a PBMC- (left panel) and a WB-based assay (right panel) in a training (1.) and a validation cohort (2.). The numbers of well-classified subjects are indicated, as well as their percentages. **(B)** Patients with aTB were discriminated from LTBI subjects by using a combination of HBHA-IFN-γ and ESAT-6-IP-10 in a PBMC-based assay (left panel) or of HBHA-IL2, HBHA-GM-CSF and ESAT-6-IP-10 in a WB-based assay (right panel) in a training (1.) and a validation cohort (2.). Only the *M. tuberculosis*-infected subjects well-classified in **(A)** were taken into account for this discrimination between aTB and LTBI. The numbers of well-classified subjects are indicated, as well as their percentages.

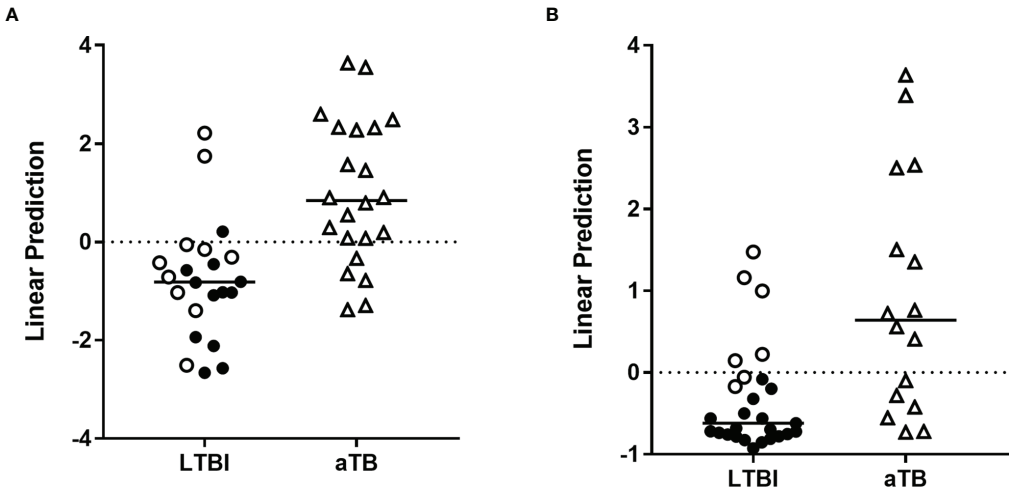

FIGURE 3 | Combination of *M. tuberculosis*-specific immune markers allowing the distinction between LTBI and aTB in the PBMC assay. HBHA-IFN-γ and ESAT-6-IP-10 were the markers selected by logistic regression analysis to be combined for the PBMC-based assay to discriminate aTB from LTBI. Patients with aTB are indicated by open triangles and LTBI subjects at risk to reactivate the infection by open circles. Results are represented with their linear predictor for the validation **(A)** and training **(B)** cohorts. The horizontal lines represent the medians and the dotted lines arbitrary cut-offs.

TABLE 2 | Demographic and clinical data of the misclassified aTB patients from the training cohort.

Patient number	Tuberculosis type	Age (years)	Sex	Country of origin	Time since arrival in Belgium	TB risk factors	Sputum smear	Mtb culture	Chest Xray
1	P	30	F	Morocco	7 years	Travels in endemic countries	Positive	Positive	Cavitation
2	P	33	M	Belgium	1 year	Prisoner in high-endemic country, Past-TB	Unknown	Positive	Suspected infiltrates
3	EP lymphadenitis	36	F	Morocco	Unknown	Contact with TB index case	Negative	Negative	Normal
4	P	64	M	Romania	6 months	Contact with TB index case, illegal, alcoholism, tobacco	Positive	Positive	Cavitation
5	EP meningitis	19	M	Morocco	Unknown	Unknown	Negative	Negative	Normal
6	EP spondylodiscitis	30	F	Ivory Coast	5 years	Pregnancy	Negative	Positive	Normal
7	EP Pleural	24	F	Italia-Morocco	4 years	Multiple sclerosis, diabetes, immunosuppressive treatment, contact with TB index case, travels to Marocco	Negative	Positive	Pleural effusion

Mtb, M. tuberculosis; P, pulmonary; EP, extrapulmonary.

TABLE 3 | Demographic and clinical data of the misclassified aTB patients from the validation cohort.

Patient number	Tuberculosis type	Age (years)	Sex	Country of origin	Time since arrival in Belgium	TB risk factors	Sputum smear	Mtb culture	Chest Xray
1	P	52	M	Pakistan	Unknown	Diabetes, homeless	Positive	Positive	Cavitations
2	P	35	M	Rwanda	22 years	Homeless, alcoholism, tobacco, denutrition	Positive	Positive*	Cavitation and infiltrates
3	EP lymphadenitis	36	F	Morocco	Unknown	Past-LTBI (1994)	unknown	Positive	Normal
4	P	43	M	Morocco	Unknown	Travels in endemic countries	Positive	Positive	Cavitation and infiltrates
5	P	63	M	Morocco	Unknown	Travels in endemic countries	Positive	Positive*	Cavitation
6	P	45	M	Poland	Unknown	Homeless, Alcoholic hepatitis/Acute cirrhosis	unknown	Positive	Bilateral nodules
7	P	45	M	Romania	1 year and 7 months	Alcoholism	Positive	Positive*	Infiltrate
8	P	46	M	Cameroun	Unknown	Unknown	unknown	Positive	Pulmonary condensation, mediastinal adenopathies

*Mtb, M. tuberculosis; P, pulmonary; EP, extrapulmonary; * positive polymerase chain reaction.*

in infected than in non-infected individuals. ROC curves analyses differentiated infected from non-infected subjects with AUCs generally higher than 0.7 (**Supplementary Table 2**). Random forest analyses of the 13 analytes secreted in response to ESAT-6 and HBHA classified HBHA-induced IL-2, TNF-α and IFN-γ, together with ESAT-6-induced IL-8, TNF-α, IP-10 and IFN-γ as the optimal markers (**Supplementary Figure 1C**).

Logistic regression analysis on the results obtained for the training cohort identified three promising combinations of markers to be used to rule-out infection: HBHA- and ESAT-6-induced IFN-γ, HBHA- and ESAT-6-induced TNF-α, HBHA-induced IL-2 and ESAT-6-induced TNF-α. The first combination provided the best results on both cohorts. Eighty-nine percent and 90% of the subjects were correctly classified as infected or not in the training and validation cohorts, respectively (**Figures 4A, B**). Five controls and two aTB were misclassified in the training cohort as well as one control, three LTBI and three aTB in the validation cohort (**Figures 2A**, **3A, B**). Three misclassified controls and two misclassified aTB patients with the WB assay were also misclassified in the PBMC assay. The clinical characteristics of the misclassified aTB patients are provided in **Table 2** (n°5 and n°6) for the training cohort and in **Table 3** (n°1, n°2 and n°7) for the validation cohort.

Selection of WB Markers to Differentiate aTB From LTBI

A second Random forest analysis was performed to identify the best markers to differentiate aTB from LTBI in the training cohort. All infected individuals were included in this analysis as a few patients with aTB were not identified as infected by the previous combination. ESAT-6-induced IP-10, and HBHA-induced IL-8, IL-2 and GM-CSF were identified as the best markers to differentiate aTB from LTBI (**Supplementary Figure 1D**). The optimal combination provided by further logistic regression was ESAT-6-induced IP-10 combined with HBHA-induced IL-2 and GM-CSF, which allowed us to correctly classify 82% of the subjects (**Figure 5A**). Among the misclassified subjects, only two were aTB patients (**Table 2**, n°4 and n°7), while the other six were LTBI subjects.

In the validation cohort, this combination correctly classified 75% of the subjects (**Figure 5B**). As for the training cohort, only two aTB patients were misclassified (**Table 3**, n°1 and n°8), whereas the other nine misclassified subjects were LTBI subjects.

Good differential diagnosis between aTB and LTBI was thus possible in most cases by combining ESAT-6-induced IP-10 to HBHA-induced IL-2 and GM-CSF. However, this combination did not allow us to identify all the aTB patients who were initially misclassified as non-infected controls. Applying the two-steps approach to first identify infected subjects and then to identify aTB patients among infected individuals resulted globally for the two cohorts in a correct identification of 31/34 aTB patients.

This combination misclassified several LTBI subjects but most of them (11/15) were considered as being at risk to reactivate their infection.

DISCUSSION

TB control could be significantly improved if simple and rapid triage or rule-out tests were available to first exclude non-infected subjects and then differentiate as much as possible aTB from LTBI. The development of triage tests designed for use by first-contact health care providers as a rule-out test of TB was reported by the World Health Organization as a high priority need for TB control (22). This strategy would limit the number of individuals requiring a confirmatory test. High sensitivity is needed for this approach to avoid missing patients with aTB. In contrast, the specificity of this triage test

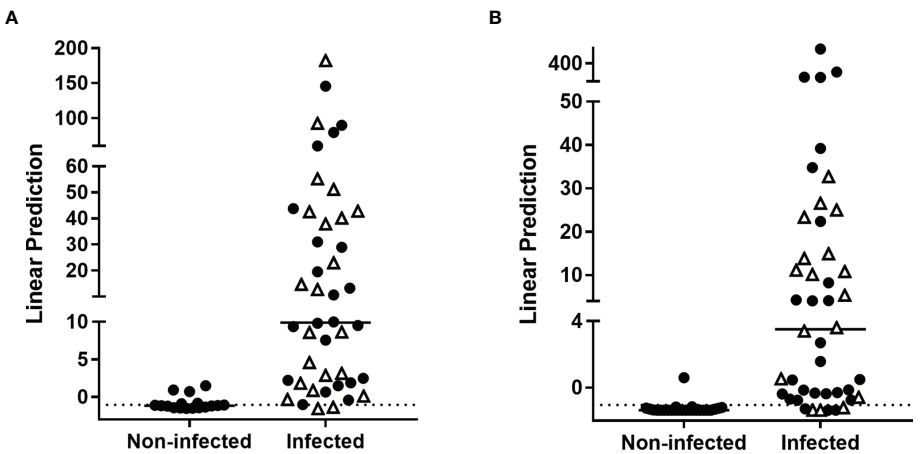

FIGURE 4 | Combination of *M. tuberculosis*-specific immune markers allowing the distinction between *M. tuberculosis*-infected versus non-infected subjects in the WB assay. HBHA-IFN-γ and ESAT-6-IFN-γ were the markers selected by logistic regression analysis to be combined for the WB-based assay to discriminate *M. tuberculosis*-infected from non-infected subjects. Patients with aTB are indicated by open triangles. Results are represented with their linear predictor for the validation **(A)** and training **(B)** cohorts. The horizontal lines represent the medians and the dotted lines arbitrary cut-offs.

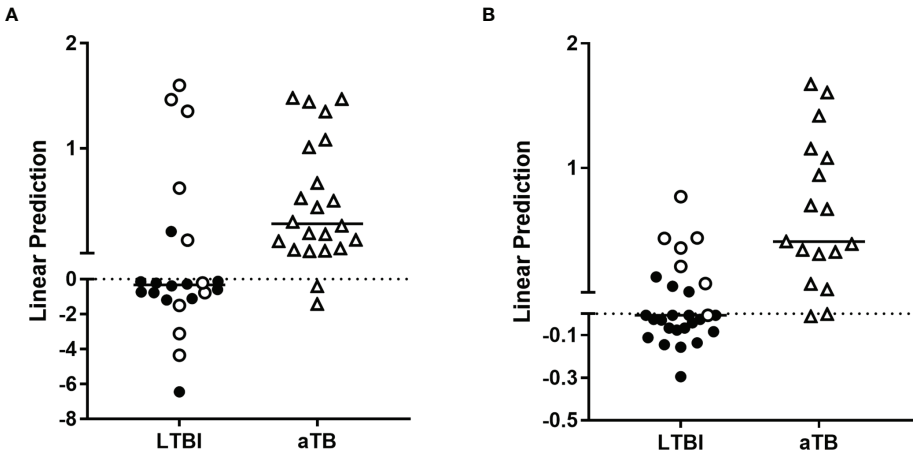

FIGURE 5 | Combination of *M. tuberculosis*-specific immune markers allowing the distinction between LTBI and aTB in the WB assay. HBHA-IL2, HBHA-GM-CSF and ESAT-6-IP-10 were the host markers selected by logistic regression analysis to be combined for the WB-based assay to discriminate aTB from LTBI. Patients with aTB are indicated by open triangles and LTBI subjects at risk to reactivate the infection by open circles. Results are represented with their linear predictor for the validation **(A)** and training **(B)** cohorts. The horizontal lines represent the medians and the dotted lines arbitrary cut-offs.

approach may be lower, as the patients initially selected by the triage test will benefit from more in-depth evaluation of their status. Blood-based immunological tests might be appropriate as triage tests. We previously reported that secretion of IFN-γ by PBMC or WB in response to *in vitro* stimulation with HBHA is a biomarker for LTBI contrasting with ESAT-6-induced IFN-γ secretion that is more characteristic of aTB (14, 15). Therefore, we suggested that combining IFN-γ secretion in response to HBHA and to ESAT-6 would help to differentiate LTBI from aTB and allows to stratify LTBI subjects in different groups associated with risks of reactivation of the infection (16, 21). In this study, we evaluated combinations of cytokines/chemokines released in response to these two antigens for improved identification of aTB patients, which could then be proposed as a triage test to select patients likely presenting aTB for further investigations to confirm the diagnosis. We propose a two-step approach consisting of first identifying infected subjects and then differentiating aTB from LTBI among infected individuals. Aiming to provide an easy test, we searched for optimal cytokine/chemokine combinations secreted by 24 hours-stimulated WB. However, this approach might not be sensitive enough for patients with lymphopenia or important inflammatory syndrome associated with high levels of plasma proteins that could inhibit cellular immune responses. It might also not be suitable for frozen material. We therefore also evaluated optimal cytokine/chemokine combinations secreted after 24 hours of *in vitro*-stimulated PBMC.

Among the 13 measured cytokines/chemokines secreted in response to HBHA and ESAT-6 and their various combinations to identify *M. tuberculosis*-infected patients, the best results were obtained both on WB and on PBMC by combining IFN-γ concentrations secreted in response to HBHA and to ESAT-6, as previously shown (15, 16). This approach allowed us to discard for further analysis most non-infected controls, 82%/71% in the training cohort and 84%/96% in the validation cohort, in the PBMC and WB assays, respectively. Misclassified controls were most often doubtful rather than being clearly positive. In addition, among the 10 misclassified controls in the PBMC and/or WB assay for both cohorts, five were health care workers potentially exposed to aTB patients, and four reported frequent travel to high TB endemic countries. Although these individuals were classified as non-infected, based on a negative TST result as recommended in Belgium (23), we cannot formally exclude that they developed immune responses to mycobacterial antigens as a consequence of exposure to *M tuberculosis*. They represented a minority of the enrolled controls that should benefit from further investigation (medical visit, chest radiograph, eventually followed by microbiological analysis of sputum samples) to exclude an ongoing *M. tuberculosis* infection. On the other hand, most infected individuals and especially most aTB patients were correctly identified and were selected for further investigations (100%/91% and 88%/82% in the training cohort in the validation cohort, in the PBMC and WB assays, respectively). Among the five misclassified aTB patients either in the PBMC or the WB assay in both cohorts, two had extra-pulmonary TB (one meningitis and one spondylodiscitis in a pregnant women) with obvious symptoms, while the other three had symptomatic pulmonary TB with severe denutrition and serious abnormalities on chest radiographs (**Tables 2** and **3**).

As a second step, we searched for the best combination of cytokines/chemokines secreted by the two stage-specific mycobacterial antigens to allow us to identify patients with aTB among infected individuals. Results were slightly different between the PBMC and WB assays. For PBMC stimulation, the best combination was HBHA-induced IFN-γ and ESAT-6-induced IP-10 that allowed us to correctly identify 77% and 71% of aTB patients in the training and validation cohorts, respectively. The interest of ESAT-6-induced IP-10 for diagnosis of *M. tuberculosis*

infection was previously reported but with little added value compared to IFN-γ, except for patients with immune deficiencies (24). ESAT-6-induced IP-10 was not reported to provide differential diagnosis between aTB and LTBI. However, we show here that, when combined with HBHA-induced IFN-γ, it improves the differentiation between aTB and LTBI. This differentiation allowed us to identify ¾ of the patients with aTB, which is an improvement over previous studies. In addition, most misclassified aTB patients were shown to be infected in the first stage analysis and will therefore receive clinical attention.

Globally, eight LTBI subjects were misclassified as aTB. Seven of them were considered in a previous study as being at risk to reactivate their infection, based on their high ESAT-6-induced IFN-γ secretion (21). Latency is considered as a spectrum of different stages of activity of persistent mycobacteria, from totally quiescent to persistently multiplying (17, 18). This latter stage is the highest risk stage and therefore may not be possible to be differentiated from subclinical TB using immunological biomarkers. We may therefore hypothesize that these 8/49 misclassified LTBI subjects might have subclinical TB. Therefore, these individuals should be prioritized for more extensive evaluation for a possible aTB.

For WB stimulation, the best discrimination between aTB and LTBI was obtained by combining HBHA-induced IL-2 and GM-CSF with ESAT-6-induced IP-10. This allowed us to identify 90% and 93% of the aTB patients among infected individuals from the training and validation cohorts, respectively. The main advantages of the WB assay are the relatively small blood volume required (feasible on 1 mL WB) and easiness to perform. The good sensitivity should allow us to propose these tests as a triage test for aTB as the recommended sensitivity is to be at least 90% (22), and should help to limit the number of individuals who require confirmatory tests. One third of the LTBI subjects were misclassified and would require the confirmatory test. However, as for LTBI subjects misclassified with the PBMC assay, most of them were considered at risk to reactivate the infection and should therefore be prioritized for further evaluation of their status.

The sensitivities achieved with these combinations are similar to those reported in a few other studies aiming to define combinations of biomarkers that provide diagnostic test accuracy consistent with WHO specifications for a rule-out test for aTB. These studies that were recently summarized (25), are based on combinations of various serum markers, mostly cytokines/chemokines (26–28), sometimes combined with antibodies against a TB antigen to raise the specificity for TB (29). We chose in our study to measure cytokines/chemokines released in response to mycobacterial antigens to increase the specificity for TB. A few studies already addressed this question by measuring chemokines/cytokines induced by the peptides from the QuantiFERON, with different marker selections among the studies (13). Our results are difficult to compare to these studies, as we selected combinations of chemokines/cytokines induced by two different stage-specific mycobacterial antigens, ESAT-6 and HBHA, in order to cover a wide range of the *M. tuberculosis* metabolism. ESAT-6 is highly expressed during bacterial multiplication, while HBHA is a latency-associated antigen, whose gene is upregulated in hypoxic conditions and in cells harboring *M. tuberculosis* during latency (30–32). Not surprisingly, the selected combinations to differentiate aTB from LTBI were different between the PBMC and WB assays, since during acute inflammation plasma proteins may modify cellular immune responses.

Strengths of our study are that the biomarkers identified in the training cohort were validated in an independent validation cohort, both for the PBMC and the WB assays, and that these biomarkers are easy to measure in most laboratories. In addition, as blood and not sputum-based biomarkers, they are suitable to identify both pulmonary and extra-pulmonary TB who were both included in our cohorts. A limitation of this study results from the relatively low numbers of individuals in each cohort and from the heterogeneity of the cohorts that comprised both patients with pulmonary and extra-pulmonary TB. Moreover, we did not include in our cohorts TB-like diseases, which may perhaps represent more relevant negative controls for the evaluation of biomarkers to diagnose aTB. Inclusion of TB-like diseases was unfortunately not possible, because no screening for LTBI is performed in these patients in Belgium so that correct classification of these patients would have been difficult.

Based on the results of this pilot study performed in a low TB incidence country, we propose a two-steps algorithm to identify patients who are highly suspected to present aTB and should be further investigated (**Figure 2**). This algorithm warrants further evaluation on larger cohorts of subjects both in low and high TB endemic countries to confirm its robustness as triage test for aTB. Albeit not as simple as recommended to be a point of care test (22), it has the advantage of being relatively easy to perform on small blood volumes, to provide acceptable identification of aTB patients, and to be less expensive than systems biology approaches that have identified diagnostic signatures to discriminate aTB from LTBI (33, 34).

DATA AVAILABILITY STATEMENT

The raw data supporting the conclusions of this article will be made available by the authors, without undue reservation.

ETHICS STATEMENT

The studies involving human participants were reviewed and approved by Ethics Committee of ULB-Hôpital Erasme, Brussels, Belgium. The patients/participants provided their written informed consent to participate in this study.

AUTHOR CONTRIBUTIONS

VD designed the study, analyzed the data and wrote a first draft of the manuscript. PC performed the complete statistical analysis of the data. AP performed all the experimental work. MH, ND, and SA included patients, provided clinical data and reviewed the manuscript. JR did the initial statistical analysis. MS prepared ESAT-6 antigens. CL coordinated the preparation of antigens

and critically reviewed the manuscript. FM designed the study, interpreted the data, and drafted the final manuscript. VC designed the study, analyzed and interpreted the data, collected clinical data, drafted figures and tables and critically reviewed the manuscript. All the authors contributed to this article and approved the submitted version.

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SUPPLEMENTARY MATERIAL

The Supplementary Material for this article can be found online at: https://www.frontiersin.org/articles/10.3389/fimmu.2022.842604/full#supplementary-material

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Improving Assignments for Therapeutic and Prophylactic Treatment Within TB Households. A Potential for Immuno-Diagnosis?

Dhanasekaran Sivakumaran[1,2], *Synne Jenum*[3*], *Christian Ritz*[1,4], *Mario Vaz*[5,6], *Timothy Mark Doherty*[7] *and Harleen M. S. Grewal*[1,2*]

[1] Department of Clinical Science, Bergen Integrated Diagnostic Stewardship Cluster, Faculty of Medicine, University of Bergen, Bergen, Norway, [2] Department of Microbiology, Haukeland University Hospital, University of Bergen, Bergen, Norway, [3] Department of Infectious Diseases, Oslo University Hospital, Oslo, Norway, [4] National Institute of Public Health, University of Southern Denmark, Copenhagen, Denmark, [5] Department of Physiology, St. John's Medical College, Bangalore, India, [6] Division of Health and Humanities, St. John's Research Institute, Bangalore, India, [7] GlaxoSmithKline Vaccines, Wavre, Belgium

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Novel N. Chegou,
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Norman Nausch,
Deutsche Gesellschaft für Internationale Zusammenarbeit, Germany

***Correspondence:**
Harleen M. S. Grewal
Harleen.Grewal@uib.no
Synne Jenum
synjen@ous-hf.no

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Delays in diagnosis and treatment of pulmonary tuberculosis (TB) can lead to more severe disease and increased transmission. Contact investigation among household contacts (HHCs) of TB patients is crucial to ensure optimal outcomes. In the context of a prospective cohort study in Palamaner, Southern India, this study attempted to assess the potential of 27 different soluble immune markers to accurately assign HHCs for appropriate treatment. A multiplex bead assay was applied on QuantiFERON (QFT)-nil supernatants collected from 89 HHCs grouped by longitudinal QFT status; *M. tuberculosis* (*Mtb*) infected (QFT positive at baseline and follow-up, n = 30), recent QFT converters (QFT-negative at baseline, n = 27) and converted to QFT-positivity within 6 months of exposure (at follow-up, n = 24) and QFT consistent negatives (n = 32). The 29 TB index cases represented Active TB. Active TB cases and HHCs with *Mtb* infection produced significantly different levels of both pro-inflammatory (IFNγ, IL17, IL8, IP10, MIP-1α, MIP1β, and VEGF) and anti-inflammatory (IL9 and IL1RA) cytokines. We identified a 4-protein signature (bFGF, IFNγ, IL9, and IP10) that correctly classified HHCs with *Mtb* infection vs. Active TB with a specificity of 92.6%, suggesting that this 4-protein signature has the potential to assign HHCs for either full-length TB treatment or preventive TB treatment. We further identified a 4-protein signature (bFGF, GCSF, IFNγ, and IL1RA) that differentiated HHCs with *Mtb* infection from QFT consistent negatives with a specificity of 62.5%, but not satisfactory to safely assign HHCs to no preventive TB treatment. QFT conversion, reflecting new *Mtb* infection, induced an elevated median concentration in nearly two-thirds (19/27) of the analyzed soluble markers compared to the levels measured at baseline. Validation in other studies is warranted in order to establish the potential of the immune biosignatures for optimized TB case detection and assignment to therapeutic and preventive treatment of *Mtb* infected individuals.

Keywords: *Mtb* infection, protein signature, active TB, preventive therapy, soluble protein markers, cytokine and chemokines

INTRODUCTION

Even before the impact of the COVID-19 pandemic on health system capacity, the decline in TB incidence by 2019 was already too slow to reach the first milestone of the End TB Strategy, with still an estimated number of 10.0 million new TB cases annually (1). The COVID-19 pandemic has impacted everyday life, damaged economies, and overwhelmed health care systems (2, 3). The interruption of health services to a point where identification of new TB cases and treatment initiation decreased by 25–50% over 3 months (between April and June, 2020) may upsurge TB deaths by 0.2–0.4 million globally in 2020 alone (1). Notably, early identification and treatment is a priority of the End TB Strategy to minimize transmission mandatory to reach the goal of eliminating the TB epidemic (4, 5).

The risk of TB progression is highest in newly exposed subjects; thus, active case finding in the households of newly diagnosed TB index patients is considered an effective strategy (6). The purpose of contact investigations is to identify subjects with increased risk of TB progression in order to offer preventive TB treatment (7). The WHO recommendations for preventive TB treatment depend on the national health resource setting and the epidemiological context. In low- and middle-income countries, preventive TB treatment is recommended for high-risk subjects such as children aged below 5 years and people living with HIV or other immunosuppressant conditions. Active TB must be ruled out by assuring the absence of the hallmark TB symptoms; cough, fever, weight loss and night sweats (8). In order to achieve early case identification and avoid TB progression with the risk of subsequent transmission, TB control programs could profit from more ambitious goals: To identify all household contacts with active/subclinical TB or *M. tuberculosis* (*Mtb*) infection at risk of TB progression, and, dependent on the condition, assign appropriate treatment. For active/subclinical TB (drug-sensitive *Mtb* isolates) this would be a full 6-month course (2-month intensive phase; isoniazid, rifampicin, pyrazinamide, and ethambutol) followed by a 4-month continuation phase; isoniazid, rifampicin), and for *Mtb* infected a prophylactic course of 3 months (isoniazid, rifampicin/rifapentine) either once-weekly or daily, or 9 months (isoniazid) either twice-weekly or daily (9). Accurate assignment of patients is vital to avoid under- and over-treatment, risking either relapse and/or *Mtb* resistance in the case of undertreating, or side-effects without gain in the case of overtreating (10).

It has repeatedly been shown that despite heavy and prolonged *Mtb* exposure, a certain proportion of subjects does not develop detectable T cell responses (for e.g., they remain QFT negative through repeated testing). These subjects have the lowest risk of TB progression, suggesting that they possess effective natural TB immunity, likely mediated by innate immune mechanisms (11, 12). No proxy marker or biosignature for this kind of natural protective immunity has been established, but finding such a biomarker or biosignature is considered the holy grail within TB immunology (13): Immunobiomarkers in TB may be useful to understand the role of host responses in natural protection or vulnerability towards *Mtb* infection, but also hold a potential for new diagnostic tools and proxy-readouts for vaccine and/or treatment efficacy (14).

In the setting of a longitudinal study of index TB cases and their household contacts, the Household Contact Cohort (HHC) study, conducted in Palamaner, Southern India, in the period 2010–2012, aimed to explore the potential of 27 soluble immune markers detected in QuantiFERON (QFT) nil tube supernatants to accurately assign TB-exposed subjects within a household to either i) standard TB treatment (concomitant active and/or subclinical TB cases) or ii) preventive treatment (*Mtb* infected with risk of TB progression) (15) or eventually iii) no treatment in subjects that likely possess natural immunity. Furthermore, we aimed to explore if the selected markers could provide some insight into natural TB immunity.

METHODS AND MATERIALS

Ethical Consideration

Ethical approval for this study was obtained from the Institutional Ethical Review Board (IERB) of St. John's Medical College, Bangalore (IERB/1/527/08). The material transfer agreement between St. John's Medical College, Bangalore, and the University of Bergen, Norway, was obtained from the Department of Biotechnology, Government of India (No. BT/Med.II/Adv (SS)/Misc./02/2012). Ethical approval was also obtained from Western Norway's Regional Committee for Medical and Health Research Ethics (Ref no: **2018/1614 D**).

Study Population

The present study is a cross-sectional study nested within a prospective cohort study of adult PTB index cases and their household contacts and conducted at our study site at the Emmaus Swiss Leprosy Project and Referral Hospital, Palamaner and Kuppam Taluks, Chittoor district, Andhra Pradesh, India (3.200°N, 72.7500°E, altitude 683 m) between September 2010 and April 2012. In total, 176 pulmonary TB (PTB) index cases were identified at the microscopy centers of the Revised National Tuberculosis Control Program (RNTCP) run by the Government of India and 164 recruited after written informed consent. In the context of our study, TB was confirmed in 150 cases by the presence of *Mtb* in sputum smear and/or culture. Positive cultures were confirmed by using the GenoType MTBC kit (Hain Life science GmBH, Nehren, Germany). Direct PCR by COBAS Taqman MTB test (Roche Diagnostics Ltd, Rotkreuz, Switzerland) was undertaken on culture-negative specimen for patients with chest X-ray (CXR) finding suggestive of TB. Recruited PTB index cases were treated with standard anti-TB treatment (ATT) and followed until completion of the 6-month treatment course. A total of 525 household contacts of the 176 PTB index cases were recruited. Household contacts who were not part of the 150 *Mtb* culture-positive index TB cases were also included for the investigation. Written informed consent was provided by all adults. For children ≤7 years, parents/guardians provided written

informed consent, and for participants >7 years, an additional written assent was obtained. The intended follow-up for all household contacts was one year.

Clinical Assessments and Sampling

Baseline assessments of PTB index cases and household contacts: Medical history (namely, BCG vaccination status, history of TB exposure, prior TB/ATT, socio-economic status, and risk factors such as diabetes, smoking, and alcohol consumption), socio-demographic, anthropometric, and clinical data were recorded. At baseline, a tuberculin skin test (TST) was performed by a trained nurse (2 TU/0.1 ml tuberculin; Span Diagnostics, Surat, India) and read after 48–72 h; an induration >10 mm was defined as positive. Blood 3 ml was collected for the QuantiFERON-TB Gold In-tube (QFT-GIT) assay and the supernatants (plasma) from the Nil, TB antigen (ag) and Mitogen tubes were collected in two microfuge tubes and stored at −80°C. One microfuge tube was used for the QFT-GIT assay and the other tube was stored for Bio-Plex assay. Three independent radiologists interpreted the CXR (anteroposterior view) at baseline, and agreement by at least two was required for the assignment abnormal TB chest X-ray. CXR was again done at the end of the study closure for all participants. Since the prevalence of HIV in India is low [<0.5%] (16), after pre-test counseling, only participants who volunteered for HIV testing were tested. Agreement for HIV testing was not a criterion for inclusion.

Sample Selection and Definition of Groups

PTB index cases were selected to represent Active TB disease (Group 1) as concomitant cases in the household were referred to the RNTCP and excluded from the HHC. Stored blood samples available from 109 of 150 *Mtb* culture-positive participants, and only 48 of 109 blood samples were obtained before ATT initiation for QFT, of which only 39 index TB cases had either previously diagnosed TB >3 years or no prior TB reported. These 39 samples were considered to include in this study. However, QFT Nil tube samples were available for 29 of 39 PTB index cases and constituted Group 1, Active TB disease.

Household contacts being QFT positive at baseline and at repeated testing at 2-, 6-, and 12-month follow-up and did not develop TB disease, were assigned as QFT consistent positive *Mtb* infected (Group 2) and were considered representative of historical and/or likely recent *Mtb* exposure.

Household contacts, who were QFT negative at baseline but converted to QFT positivity at 2- or 6-month follow-up and did not develop TB disease, were assigned to recent QFT converters (Group 3) and were considered representative of recent *Mtb* exposure.

Household contacts that remained QFT negative throughout the 12-month follow-up and that did not develop TB were assigned to QFT consistent negatives (Group 4) and were considered representative of likely natural protective innate mechanisms towards TB.

Both *Mtb* infected (QFT consistent positive) and recent QFT converters (QFT positivity at 2- or 6-month follow-up) were considered representative of contacts that would profit from preventive treatment without risk of undertreating subclinical TB/active TB. Therefore, these two groups were merged and called Household contacts with *Mtb* infection.

Sample selection is shown in **Figure 1**. For the purpose of this study, Active TB (n = 29), QFT consistent positive *Mtb* infected (n = 30; hereafter referred to as *Mtb* infected throughout this manuscript), recent QFT converters (n = 27 who were QFT-negative at baseline but who converted to QFT-positivity within 6 months of exposure; 14 converted at 2 months and 10 converted at 6 months and for 3 samples the follow-up Nil-ag tubes were not available) and QFT consistent negatives (n = 32). In total, 142 samples were included for the biomarker analysis.

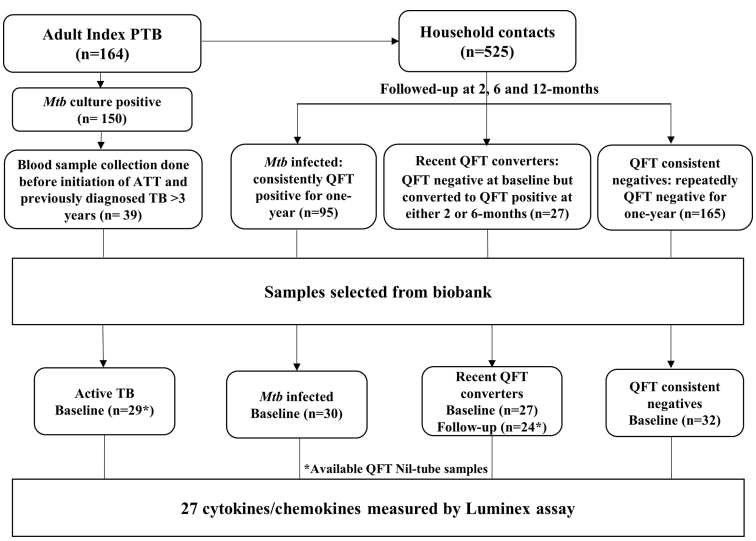

FIGURE 1 | Study flow chart.

Multiplex Cytokine/Chemokine Assays

For the Bio-Plex Multiplex Immunoassays, we used the Nil tube supernatants from the QFT-GIT assay. This unstimulated control sample can be used as a substitute for separate serum stored samples (17). The selected 142 samples were tested using the 'Human cytokines 27-plex' kit (Bio-Rad Laboratories Inc., Hercules, CA, USA), according to the instructions of the manufacturer. The 27 biomarkers included are Interleukin (IL)-1β, IL-1 receptor antagonist (IL1-ra), IL-2, IL-4, IL-5, IL-6, IL-7, IL-8/CXCL8, IL-9, IL-10, IL-12 (p70), IL-13, IL-15, IL-17, eotaxin/CXCL11, basic fibroblast growth factor (bFGF), granulocyte-colony stimulating factor (G-CSF), granulocyte–macrophage colony-stimulating factor (GM-CSF), interferon (IFN)-γ, interferon-inducible protein (IP-10)/CXCL10, monocyte chemotactic protein (MCP-1)/CCL2, macrophage inflammatory protein (MIP)-1α/CCL3, MIP-1β/CCL4, platelet-derived growth factor-BB (PDGF-BB), regulated upon activation T cell expressed and secreted (RANTES)/CCL5, tumor necrosis factor (TNF), and vascular endothelial growth factor (VEGF). Data acquisition was performed on a Luminex100 analyzer (Luminex Corporation, Austin, Texas, USA) according to the instructions of the manufacturer. Cytokine/Chemokine concentrations were measured in (pg ml^{-1}).

Data Analysis

Patient characteristics were summarized as mean (standard deviation), median (interquartile range; IQR), and counts, as appropriate. Between-group comparisons of biomarker levels across participant groups (Active TB (Group 1), *Mtb* infected (Group 2), recent QFT converters (Group 3), and QFT consistent negatives (Group 4) were carried out applying the Kruskal–Wallis using Dunn's *post-hoc* test. Within-group comparisons of biomarker levels between baseline and follow-up samples carried out applying Wilcoxon matched paired test.

Analyses aiming to identify diagnostic biosignatures for which a two-step approach was applied (18). In brief, we first applied logistic regression selecting biomarkers for the Lasso regression analysis. A predicted probability >0.5 resulted in classification as participants with *Mtb* infection/disease and <0.5 as participants without *Mtb* infection/disease. The sensitivity and specificity for the identified signatures were defined by their ability to assign a correct high probability (above 0.5) to participants as either TB cases or controls. The diagnostic abilities of the signatures were summarized by means of receiver operator characteristic (ROC) curves (area under the curve [AUC], sensitivity, and specificity). Analyses were carried out using R (R Core Team, 2019) (19) through the interface RStudio (www.rstudio.com).

RESULTS

Characterization of the Study Participants at Baseline

A total of 118 study participants were classified into four groups; Active TB (n = 29), *Mtb* infected (n = 30), recent QFT converters (baseline n = 27; follow-up n = 24), and QFT consistent negatives (n = 32). The mean age was 45.6 years (SD± 13.9) for Active TB, 22.0 years (SD± 15.3) for *Mtb* infected, 29.5 years (SD± 19.8) for recent QFT converters, and 18.3 years (SD± 16.9) for QFT consistent negatives. Males constituted 79.3% (23/29) of Active TB, 36.6% (11/30) of *Mtb* infected, 48.1% (13/27) of recent QFT converters, and 50.0% (16/32) of QFT consistent negatives. As expected, the groups with negative QFT at baseline (QFT converters and QFT consistent negatives) all had a TST <10 mm. Therefore, the only meaningful comparison was between Active TB (median TST 15 mm) and *Mtb* infected (median TST 14.5 mm). Interestingly, but consistent with reduced QFT sensitivity in active TB, median IFNγ concentration was higher in *Mtb* infected compared to Active TB. Complete clinical characteristics are shown in **Table 1**.

Cytokines Patterns in Active TB Cases and Exposed Household Contacts at Baseline

Twenty-seven different cytokines, including chemokines and markers of inflammation, were analyzed in QFT-GIT Nil tube supernatants, and compared between Active TB cases and household contacts grouped by *Mtb* infection status following exposure as described. Compared to Active TB, *Mtb* infected exhibited higher levels of the inflammatory T-cell cytokines IFNγ, IL9, and IL17, and the vascular endothelial growth factor (VEGF), whereas the pro-inflammatory IP10, released from innate cells in response to IFNγ was higher in Active TB (**Figure 2**).

Compared to Active TB, QFT consistent negatives had lower levels of the innate pro-inflammatory IL8 promoting neutrophil chemotaxis, macrophage inflammatory protein (MIP)-1α and -1β and IP10, and the anti-inflammatory interleukin 1 receptor antagonist (IL1RA) that inhibits IL-1 effects. Compared to *Mtb* infected, QFT consistent negatives had lower levels of the innate pro-inflammatory cytokine granulocyte colony-stimulating factor (GCSF). QFT consistent negatives also exhibited lower levels of the anti-inflammatory IL1RA compared to Active TB and *Mtb* infected (**Figure 2**).

Dynamics in Cytokine Patterns in Recent QFT Converters

Compared to baseline, the concentrations of 19 out of 27 cytokine/chemokine markers were significantly elevated in recent QFT converters at conversion that occurred within 6 months of continuous exposure to index TB cases (**Figure 3**). All these 19 cytokine/chemokine markers play a role in the development of inflammatory and protective immune responses to microbial invaders by modulating immune cells of both the innate and adaptive immune systems. Notably, all the 19 markers were elevated in the recent QFT converter follow-up samples as compared to the other groups (**Supplementary Table 1**).

Protein Signatures With a Potential to Discriminate Active TB From *Mtb* Infected and Recent QFT Converters

To identify a protein signature associated with Active TB, univariate feature selection was applied using logistic regression (18), and identified a 6-protein signature, comprising bFGF, GCSF, IFNγ, IL9, IP10, and MIP1α (**Table 2A**) that differentiated Active TB from

TABLE 1 | Clinical Characteristics of the study groups.

Clinical Characteristics	Active TB (ATB; n = 29)	QFT consistent positives *Mtb* infected (n = 30)	Recent QFT converters baseline samples (n = 27)	Recent QFT converters follow-up samples (n = 24)	QFT consistent negatives (n = 32)
Demographics					
Mean age in years (SD)	45.6 (± 13.9)	22.0 (± 15.3)	29.5 (± 19.8)	–	18.3 (± 16.9)
Gender (Male)	23	11	13	–	16
Mycobacterial exposure					
Known BCG vaccination	11	23	17	–	23
Unknown	3	0	3	–	3
Tuberculin skin test					
Positive (>10 mm)	24	30	0	–	0
Median (mm)	15	14.5	8	–	6
QuantiFERON Gold in tube					
Positive (≥0.35 IU/ml)	23	30	0	27	0
Test result not available	1[T]	0	0	0	0
Median IFNγ (IU/ml)	3.02	9.7	0.05	0.88	0.02
Symptoms					
Cough ≥2 weeks	26	0	0	–	0
Fever ≥1 week	21	0	0	–	0
Weight loss	20	0	0	–	0
Findings					
Abnormal Chest X-ray	28	0	0	–	0
HIV test done	12	19	20	–	20
HIV positive	1	0	0	–	0
BMI <18.5 (underweight)	22	14	8	8	12

[T] *The QTF test was performed, and the test result was not available, but the QTF NIL tube was used for biomarker analysis.*

Mtb infected with an AUC of 0.89 (95% CI, 0.81–0.98; **Figure 4A**), sensitivity of 79.3% (95% CI, 60.3–92.0) and specificity of 83.3% (95% CI, 65.3–98.9) (**Table 2B**). Similarly, a 5-protein signature was identified, comprising IFNγ, IL1RA, IL9, IP10, and MIP1α that differentiated Active TB from recent QFT converters with an AUC of 0.87 (95% CI, 0.76–0.96; **Figure 4B**), sensitivity of 75.9% (95% CI, 56.5–89.7) and specificity of 81.5% (95% CI, 61.9–94.4). Interestingly, 4 proteins (IFNγ, IL9, IP10, and MIP1α) overlapped between the two identified signatures, suggesting differential expression of these is associated with active TB disease, rather than infection *per se*.

Accuracy of Protein Signatures to Assign Therapeutic or Prophylactic TB Treatment

Next, we merged household contacts that could profit from preventive TB treatment, namely *Mtb* infected, sampled at baseline, and recent QFT converters, sampled at the time of/after QFT conversion. This group, called Household contacts with *Mtb* infection (merged *Mtb* infected + recent QFT converters), was then compared against Active TB to identify a protein signature. Univariate feature selection was followed by lasso regression analysis. A 4-protein signature was identified, comprising bFGF, IFNγ, IL9, and IP10 (**Table 3A**) that differentiated Active TB from Household contacts with *Mtb* infection with an AUC of 0.89 (95% CI, 0.80–0.96; **Figure 5A**), sensitivity of 72.4% (95% CI, 52.8–87.3) and specificity of 92.6% (95% CI, 82.1–97.9) (**Table 3B**).

Protein Signatures Distinguishing QFT Consistent Negatives From Active TB and *Mtb* Infected

QFT consistent negatives were satisfactorily discriminated from Active TB by a 4-protein signature, comprising Eotaxin, IL1RA, IL8, and IP10 that differentiated with an AUC of 0.88 (95% CI, 0.80–0.97; **Figure 4C**), sensitivity of 86.2% (95% CI, 68.3–96.1), and specificity of 81.3% (95% CI, 63.6–92.8). In addition, QFT consistent negatives could to some extent be discriminated from *Mtb* infected by another 4-protein signature comprising bFGF, GCSF, IFNγ, and IL1RA (**Table 2A**) with an AUC of 0.72 (95% CI, 0.59–0.85; **Figure 4D**), sensitivity of 50.0% (95% CI, 31.3–68.7), and specificity of 71.9% (95% CI, 53.3–86.3) (**Table 2B**). Notably, IL1RA overlapped between the two identified signatures.

Accuracy of Protein Signatures to Safely Assign no TB Treatment

Next, Household contacts with *Mtb* infection (merged *Mtb* infected + recent QFT converters) were compared to QFT consistent negatives by lasso analysis. A 4-protein signature

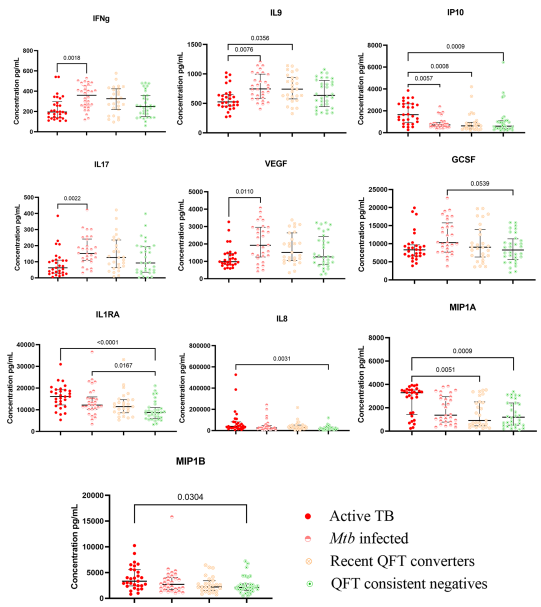

FIGURE 2 | Kruskal–Wallis test with Dunn's post correction was applied. Scatter-plot graph depicting median cytokine concentrations (pg ml^{-1}) from the QFT-GIT supernatants (nil tube) by the Multiplex bio-plex assay. Nil levels (pg ml^{-1}) of IFNγ, IL9, IP10, IL-17, VEGF, GCSF, IL1RA, IL8, MIP1α, and MIP1β in patients with active TB (ATB; n = 29), *Mtb* infected (n = 30), recent QFT converters (n = 27), and QFT consistent negatives (n = 32).

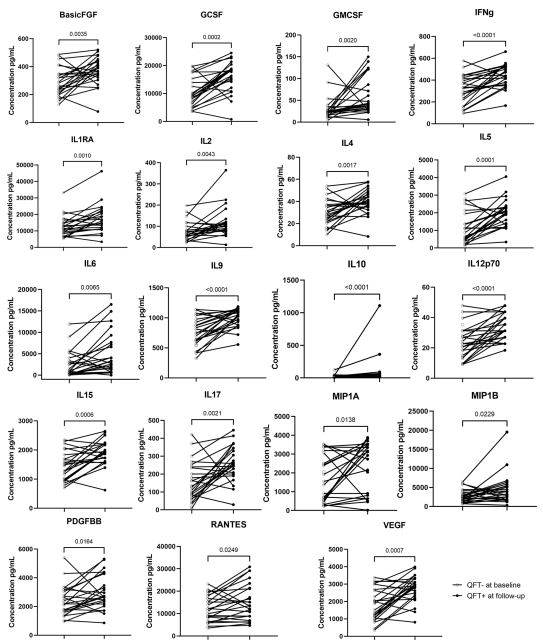

FIGURE 3 | Wilcoxon matched paired test was applied. Symbols and lines graph depicting individual changes in cytokines/chemokines concentrations (pg ml^{-1}) from recent QFT converters in baseline samples (n = 27) to recent QFT converters in the follow-up sample (n = 24).

was identified, comprising bFGF, GCSF, IFNγ, and IL1RA (**Table 3A**) that differentiated Household contacts with *Mtb* infection from QFT consistent negatives with an AUC of 0.81 (95% CI, 0.72–0.90; **Figure 5B**), sensitivity of 87.0% (95% CI, 75.1–94.6), and specificity of 62.5% (95% CI, 43.7–78.9; **Table 3B**) for safe assignment to no TB treatment.

DISCUSSION

The natural history of TB is characterized by a dynamic spectrum of disease, in which newly infected individuals are at the highest risk of developing active disease, but may also enter a state of long-term latent infection from which they may progress to disease many years after infection (20). Understanding where infected individuals are in this spectrum from infection to disease progression is the key to appropriate management and treatment. The purpose of contact investigations following identification of a TB index case is early case detection to limit morbidity and further transmission. This can be achieved by i) identification of concomitant TB cases in need of a therapeutic course of TB treatment, and ii) identification of individuals with recently acquired *Mtb* infection and subsequent elevated risk of TB progression who would benefit from preventive therapy or attentive clinical surveillance (21). We identified a 6-protein signature (bFGF, GCSF, IFNγ, IL9, IP10, and MIP1α) that distinguished Active TB from *Mtb* infected, and a 4-protein signature (Eotaxin, IL1RA, IL8, and IP10) that distinguished Active TB from QFT consistent negatives with AUCs of 0.89 and 0.88, respectively.

TABLE 2A | Expression and regression coefficients for each biomarker of the identified protein signature.

ATB vs. *Mtb* infected		ATB vs. Recent QFT converters		ATB vs. QFT consistent negatives		*Mtb* infected vs. QFT consistent negatives	
Cytokines	**Slope co-efficient***	**Cytokines**	**Slope co-efficient***	**Cytokines**	**Slope co-efficient***	**Cytokines**	**Slope co-efficient***
bFGF	-7.071	IFNγ	-4.101	Eotaxin	3.472	bFGF	0.868
GCSF	-0.192	IL1RA	0.064	IL1RA	0.173	GCSF	0.073
IFNγ	-0.636	IL9	-1.073	IL8	0.004	IFNγ	0.333
IL9	-1.452	IP10	0.470	IP10	0.331	IL1RA	0.076
IP10	1.021	MIP1α	0.350				
MIP1α	0.640						

**Slope coefficients are scaled-up by a factor of 1000.*

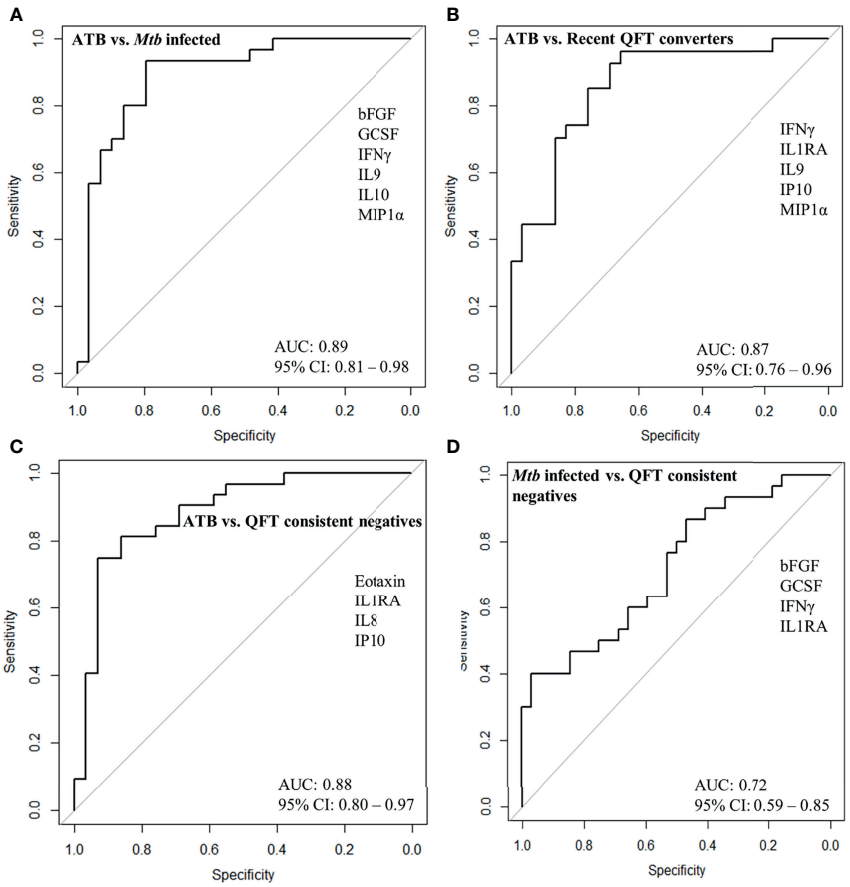

FIGURE 4 | ROC curves for protein signature that distinguishes **(A)** Active TB (ATB; n = 29) from *Mtb* infected (n = 30), **(B)** ATB (n = 29) from recent QFT converters (n = 27), **(C)** ATB (n = 29) from QFT consistent negatives (n = 32), and **(D)** *Mtb* infected (n = 30) from QFT consistent negatives (n = 32).

In order to guide the assignment of HHCs to full-length TB treatment (Active TB) or preventive TB treatment (*Mtb* infected), we compared Active TB vs. Household contacts with *Mtb* infection (merged *Mtb* infected + recent QFT converters) and identified a 4-protein signature (bFGF, IFNγ, IL9, and IP10) with a specificity of 92.6%. Even with an accurate biomarker signature to identify concomitant TB cases in a household, we acknowledge the need for respiratory samples and microbiological verification before initiation of TB treatment to ascertain the diagnosis and drug sensitivity. Nevertheless, a screening test for *Mtb* infection could accurately rule out concomitant TB and limit the number of HHCs in need for the sampling for a respiratory specimen and positively assigning HHCs for preventive TB treatment with low risk of undertreating concomitant incipient and subclinical TB cases. Hopefully, the screening test would also identify and secure appropriate treatment of extrapulmonary TB cases for which microbiological verification is far more challenging. Further, to identify HHCs for whom treatment could safely be withheld due to likely natural TB immunity, we compared QFT consistent negatives with Household contacts with *Mtb* infection and identified a 4-protein signature (bFGF, GCSF, IFNγ, and IL1RA) that correctly classified QFT consistent negatives with an AUC of 0.81 and a disappointing specificity of 62.5%. Interestingly, bFGF and IFNγ overlapped between the two identified signatures. In TB pathology, T cells produce IFNγ, which aids in protective immunity against *M.*

TABLE 2B | Identification and performance of protein signature.

	Protein signature	Sensitivity in % (95% CI)	Specificity in % (95% CI)	AUC (95% CI)
ATB vs. *Mtb* infected	6-protein signature	79.3 (60.3–92.0)	83.3 (65.3–98.9)	0.89 (0.81–0.98)
ATB vs. QFT recent converters	5-protein signature	75.9 (56.5–89.7)	81.5 (61.9–94.4)	0.87 (0.76–0.96)
ATB vs. QFT consistent negatives	4-protein signature	86.2 (68.3–96.1)	81.3 (63.6–92.8)	0.88 (0.80–0.97)
***Mtb* infected vs. QFT consistent negatives**	4-protein signature	50.0 (31.3–68.7)	71.9 (53.3–86.3)	0.72 (0.59–0.85)

TABLE 3A | Expression and regression coefficients for each biomarker of the identified protein signature.

ATB vs. Household contacts with *Mtb* infection		Household contacts with *Mtb* infection vs. QFT consistent negatives	
Cytokines	**Slope co-efficient***	**Cytokines**	**Slope co-efficient***
bFGF	-3.92	bFGF	1.182
IFNγ	-2.37	GCSF	0.078
IL9	-1.90	IFNγ	1.978
IP10	0.401	IL1RA	0.074

**Slope coefficients are scaled-up by a factor of 1000.*

tuberculosis by activating macrophages, allowing them to eliminate *Mtb* more effectively (22). Still, although essential for anti-mycobacterial responses, IFNγ alone is not sufficient, and high levels of IFNγ-producing T cells do not correlate with immune protection (23). bFGF is also associated with tissue regeneration and angiogenesis, but we observed reduced levels in Active TB compared to HHCs with *Mtb* infection (QFT consistent positives).

The innate immune response that controls *Mtb* infection starts with pathogen recognition and uptake by resident tolerant alveolar macrophages in which *Mtb* finds a niche for replication. Once the bacterial load reaches beyond "the tolerant threshold", local early innate responses are activated and start producing cytokines, chemokines, and antimicrobial molecules resulting in bacterial killing/restricted growth and initiation of adaptive immune responses (24). The relevance of exploring the biomarker potential of cytokines and chemokines, and the importance of the IFNγ cytokine in TB immunology has already been mentioned. Regarding chemokines, these are essential for the recruitment of the first line of innate immune effector cells to infection and inflammatory sites and the deployment of natural immune sentinels at mucosal barriers (25). Further, the plasma levels of chemokines have the capacity to influence host immunity to sustain the protective immune response (26). Another likely advantage is that many chemokines, namely, MCP-1, MIP1α, MIP-1β, and IP-10, in adults have been reported probably not to associate with malnutrition, a major risk factor for TB globally (27), whereas this is more uncertain with TST and QFT results. Studies have reported that IP10 is a potential biomarker for TB and contributes to restricting *Mtb* replication in host tissues (28–32). Interestingly, mRNA levels of the cytokines *CCL4* (MIP1β), *CXCL10* (IP10) have been reported to decrease following isoniazid preventive treatment (33), suggesting a broad potential for chemokine biomarker tools in diagnosis and prediction of progression and evaluation of treatment response (34–36).

In our study, QFT conversion was indeed reflected in alterations in the analyzed soluble markers as nearly two-thirds (19/27) had increased median concentration from baseline to the time of/after QFT conversion (recent QFT converters). This provides insight in the early chemokines/cytokines-driven inflammatory response induced by *Mtb* infected/activated macrophages that result in recruitment and activation of innate and adaptive immune cells to the lung (26, 37). In response to granulocyte–macrophage colony-stimulating factor, monocytes, neutrophils, epithelial cells, and adipocytes produce IL-1RA (38), the level of IL1RA was elevated in Active TB in this study. A study has reported that the level of IL-1RA was higher in patients with delayed treatment response than those with a

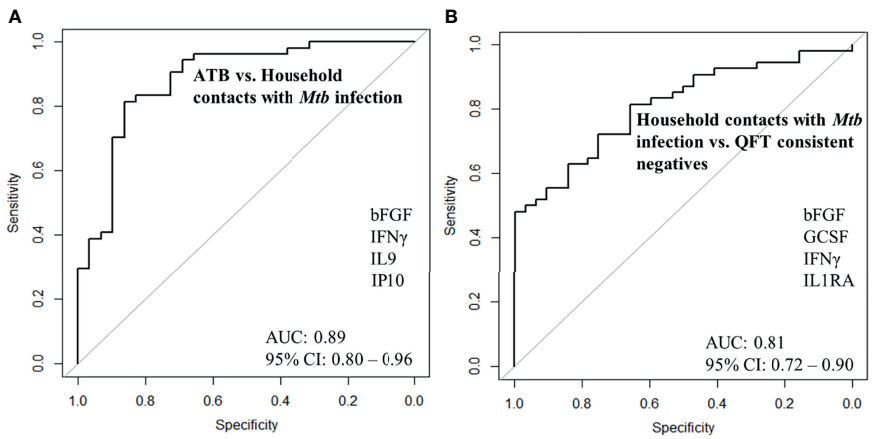

FIGURE 5 | ROC curves for signature that distinguishes **(A)** Active TB (n = 29) from Household contacts with *Mtb* infection (n = 30), **(B)** Household contacts with *Mtb* infection (n = 30) from QFT consistent negatives (n = 32).

TABLE 3B | Identification and performance of protein signature.

	Protein signature	Sensitivity in % (95% CI)	Specificity in % (95% CI)	AUC (95% CI)
ATB vs. Household contacts with *Mtb* infection	4-protein signature	72.4 (52.8–87.3)	92.6 (82.1–97.9)	0.89 (0.80–0.96)
Household contacts with *Mtb* infection vs. QFT consistent negatives	4-protein signature	87.0 (75.1–94.6)	62.5 (43.7–78.9)	0.81 (0.72–0.90)

favorable response to therapy (39). IL-9 is mainly produced by Th2 cells, promotes the pleural mesothelial cell repairing, and inhibits IFNγ-induced pleural mesothelial cell apoptosis (33). This is also consistent with a host protective response that aims to prevent excessive pathology (40). IL-9 is involved in the immune-pathogenesis of inflammatory diseases and maintaining immune tolerance, putting it in a similar role to the IL1RA. In TB, IL-17 has been suggested to protect against disease progression and pathological damage in the lung by initial neutrophil recruitment after *Mtb* infection (41). As reported in a previous study (42), the levels of IL-9 and IL-17 (measured in QFT-Nil tube plasma samples) were reduced in active TB compared to *Mtb* infection. We report that the pro-inflammatory IP10 was higher in Active TB compared to the other groups, and this finding is consistent with previous studies conducted in various populations (34, 42, 43). IP-10 also plays a chemotactic role, though it seems to be more critical for T cells. The elevated expression of these cytokines in active TB is associated with the infiltration and consolidation seen in the lungs of TB patients and suggests the elevated expression of these genes acts as a marker for TB pathology rather than *Mtb* infection (38). In the present study, the levels of chemokines IL-8, MIP-1α and MIP-1β and were elevated in active TB, IL8 and MIP-1α attract both lymphocytes and polymorphonuclear neutrophils. IL-8 is involved in the recruitment of leukocytes and the formation of granulomas. Further, MIP-1α from neutrophils may contribute to the recruitment of mononuclear cells after neutrophils have accumulated. Therefore, the production of IL-8 and MIP-1α may be greater in suppurative inflammation than in mycobacterial inflammation (39, 44).

The study reported here has some limitations, principally the fact that it analyzed a limited number of cytokines and chemokines. Moreover, the household contacts were younger than the TB index cases and there was an uneven sex distribution. Immune responses changes with age and this may have had an impact on host protein levels and the identified protein signatures. Further, the risk of TB-exposure *per se*, but also for repeated TB exposure, increases with age. By design, eligible TB index cases were adults, thereby inducing an inevitable age bias in the Active TB group. A sub-analysis undertaken in the QFT consistent negatives (n = 32), whom can be assumed to be unaffected by the probable age-dependant increase in TB exposure, did not find any significant correlation between age and cytokine concentrations. In addition, no formal sample size calculation was carried out since the maximum sample size was limited by the availability of samples for biomarker analysis and the inability to cross-validate the identified proteomic signature due to the lack of comparable samples from other cohorts. In this study, TB treatment was started in index TB cases based on recommendations, which included the presence of clinical symptoms and also microbiological and/or radiological findings. Even with a valid biomarker signature to identify concomitant TB cases in a household, we acknowledge the need for respiratory samples and microbiological verification before initiation of TB treatment to ascertain the diagnosis and drug sensitivity. Nevertheless, based on these results, further exploration of biosignatures and validation of suggested signatures in broad settings, is warranted in order to establish their potential and eventual use to guide optimal TB case detection and assignment to therapeutic and preventive treatment to *Mtb* infected individuals. Optimal individual management is currently our best option to change TB epidemiology.

DATA AVAILABILITY STATEMENT

The original contributions presented in the study are included in the article/**Supplementary Material**. Further inquiries can be directed to the corresponding authors.

ETHICS STATEMENT

Ethical approval for this study was obtained from the Institutional Ethical Review Board (IERB) of St. John's Medical College, Bangalore (IERB/1/527/08). The material transfer agreement between St. John's Medical College, Bangalore, and the University of Bergen, Norway, was obtained from the Department of Biotechnology, Government of India (No. BT/Med.II/Adv (SS)/Misc./02/2012). Ethical approval was also obtained (Ref no: 2018/1614 D) from Western Norway's Regional Committee for Medical and Health Research Ethics. Written informed consent to participate in this study was provided by the participants' legal guardian/next of kin.

AUTHOR CONTRIBUTIONS

DS, SJ, TMD, CR, and HMSG conceptualized and designed the biomarker study. MV coordinated patient recruitment and follow-up. DS and SJ wrote the manuscript with contributions from TMD, CR, and HMSG. DS performed all laboratory

experiments, data analysis and generated the tables and figures. CR supervised the statistical analysis, wrote the section on statistical analysis, and reviewed the manuscript. HMSG had primary responsibility for the final content of the manuscript. All authors listed have made a substantial, direct, and intellectual contribution to the work and approved it for publication.

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SUPPLEMENTARY MATERIAL

The Supplementary Material for this article can be found online at: https://www.frontiersin.org/articles/10.3389/fimmu.2022.801616/full#supplementary-material

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Defining Discriminatory Antibody Fingerprints in Active and Latent Tuberculosis

Nadege Nziza[1†], Deniz Cizmeci[1†], Leela Davies[1,2], Edward B. Irvine[1,3], Wonyeong Jung[1], Brooke A. Fenderson[1], Marwou de Kock[4], Willem A. Hanekom[5,6], Kees L. M. C. Franken[7], Cheryl L. Day[8], Tom H. M. Ottenhoff[7*] and Galit Alter[1*]*

[1] Ragon Institute of Massachusetts General Hospital (MGH), Massachusetts Institute of Technology (MIT) and Harvard, Cambridge, MA, United States, [2] Division of Infectious Diseases, Brigham and Women's Hospital, Boston, MA, United States, [3] Department of Immunology and Infectious Diseases, Harvard T.H. Chan School of Public Health, Boston, MA, United States, [4] South African Tuberculosis Vaccine Initiative (SATVI) and School of Child and Adolescent Health, Institute of Infectious Diseases and Molecular Medicine, University of Cape Town, Cape Town, South Africa, [5] Africa Health Research Institute, Durban, South Africa, [6] Division of Infection and Immunity, University College London, London, United Kingdom, [7] Department of Infectious Disease, Leiden University, Leiden, Netherlands, [8] Department of Microbiology and Immunology, Emory University School of Medicine, Emory University, Atlanta, GA, United States

Tuberculosis (TB) is among the leading causes of death worldwide from a single infectious agent, second only to COVID-19 in 2020. TB is caused by infection with *Mycobacterium tuberculosis* (Mtb), that results either in a latent or active form of disease, the latter associated with Mtb spread. In the absence of an effective vaccine, epidemiologic modeling suggests that aggressive treatment of individuals with active TB (ATB) may curb spread. Yet, clinical discrimination between latent (LTB) and ATB remains a challenge. While antibodies are widely used to diagnose many infections, the utility of antibody-based tests to diagnose ATB has only regained significant traction recently. Specifically, recent interest in the humoral immune response to TB has pointed to potential differences in both targeted antigens and antibody features that can discriminate latent and active TB. Here we aimed to integrate these observations and broadly profile the humoral immune response across individuals with LTB or ATB, with and without HIV co-infection, to define the most discriminatory humoral properties and diagnose TB disease more easily. Using 209 Mtb antigens, striking differences in antigen-recognition were observed across latently and actively infected individuals that was modulated by HIV serostatus. However, ATB and LTB could be discriminated, irrespective of HIV-status, based on a combination of both antibody levels and Fc receptor-binding characteristics targeting both well characterized (like lipoarabinomannan, 38 kDa or antigen 85) but also novel Mtb antigens (including Rv1792, Rv1528, Rv2435C or Rv1508). These data reveal new Mtb-specific immunologic markers that can improve the classification of ATB versus LTB.

Keywords: active and latent tuberculosis, antibodies, HIV, biomarkers, diagnostics

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Cecilia Lindestam Arlehamn, La Jolla Institute for Immunology (LJI), United States

***Correspondence:**
Galit Alter
galter@partners.org
Tom H. M. Ottenhoff
T.H.M.Ottenhoff@lumc.nl
Cheryl L. Day
cheryl.day@emory.edu

†These authors have contributed equally to this work and share first authorship

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INTRODUCTION

Infection with *Mycobacterium tuberculosis* (Mtb) affects one quarter of the world's population (WHO report 2020) and causes approximately 1.5 million deaths annually (WHO global TB report). While the majority of infected individuals control the infection for life, in a state referred to as latent tuberculosis infection (LTB) (1, 2), approximately 5-10% of the infected individuals lose control of the bacterial infection. Consequently, these individuals progress to active tuberculosis (ATB) disease, and if untreated contribute centrally to dissemination of the infection and may ultimately succumb to death (3–5). Moreover, in the setting of HIV co-infection, the risk of developing ATB is 20 to 30 times higher, and without treatment the mortality rate of ATB reaches nearly 100% (3). Thus, diagnostic tools are urgently needed to help guide clinical care across populations.

Current diagnostic tests include assays of bacteriological presence (microscopic or genetic), supplemented by the analysis of symptoms, radiological evidence and tests that involve the detection of recall-memory to Mtb antigens based on the tuberculin skin test (TST) or the ex vivo detection of memory Mtb-specific T-cells based on the interferon-γ (IFN-γ) release assay (IGRA) (6, 7). While TST and IGRA clearly capture Mtb exposure, current diagnostics distinguish ATB and LTB poorly, rendering clinical management of TB disease a major challenge in curbing deaths and disease spread. Thus, improved TB diagnostics are urgently needed to reduce morbidity and mortality.

Pathogen-specific antibodies represent critical markers of infection and disease across many infectious disorders (8–10). Since 1983, antibody responses have been studied for the serodiagnosis of TB (11–16). In addition, studies on HLA-linked immune response highlighted association between sputum smear-positive TB and expression of Class II HLA genes HLA-DR2, with HLA-DR2 being strongly correlated with antibody titers against 38-kDa Mtb protein (17), thus further motivating the identification of antibody-diagnostics to guide TB clinical care (18, 19). However, like many infectious diseases, including COVID-19 (20, 21), antibody titers to Mtb increase with pathogen burden (22–25). Because antigen load can vary drastically across subjects, antibody magnitude may be an incomplete marker of disease progression. However, emerging data strongly suggest that antibodies differ across ATB and LTB, both with respect to antigen-specificities targeted selectively across disease states, but also with respect to differences in the quality of the Mtb-specific humoral immune response (isotype, subclass, and Fc-glycosylation) (26–29). Thus far, studies aiming at developing antibody-based diagnostics for ATB and LTB have focused on a small number of Mtb antigens including lipoarabinomannan (LAM), purified protein derivative (PPD), ESAT6/CFP10, Ag85A/B and MPT64 (26, 27, 30). Even in the setting of HIV, which is associated with CD4 T cell suppression and altered humoral immunity (4), humoral immune responses to Mtb have been shown to discriminate between ATB and LTB (30). However, whether additional Mtb antigen specificities and antibody qualities could provide clearer resolution of LTB and ATB remains unclear. Thus, in this study we comprehensively profiled the humoral immune response across 209 Mtb antigens, predicted to be highly enriched in the lung under hypoxic conditions as would be present in a granuloma (31, 32). Using a systems serology approach, we identified novel classes of antigen-specific antibody profiles able to resolve and discriminate ATB and LTB, independent of HIV status.

MATERIALS AND METHODS

Study Subjects

Plasma samples from 4 groups of adults were included in this study: ATB/HIV+ (n = 12), LTB/HIV+ (n = 22), ATB/HIV- (n = 21), and LTB/HIV- (n = 22) (**Table 1**). All subjects were recruited from Cape Town, South Africa. LTB was defined as the absence of TB symptoms, no previous history of TB diagnosis or treatment and presence of IFN-γ based on an IFN-γ release assay (IGRA). ATB was defined as a positive culture for *Mycobacterium tuberculosis* (Mtb) growth or positive sputum smear microscopy. Blood from ATB individuals was obtained between 0 and 7 days of standard course anti-TB treatment following South African National Health Guidelines. None of the patients included in the study were on antiretroviral therapy (ART) at the time of enrollment. For each participant, whole blood was collected in sodium heparin Vacutainer tubes (BD Biosciences). Plasma samples were isolated by centrifugation under 500g after 5 minutes, within 4 hours of collection. Prior to the study, a written and informed consent was given by all study participants. Consents were approved by the Human Research Ethics Committee of the University of Cape Town and the Western Cape Department of Health, as well as the study institutional review board at Massachusetts General Hospital and Partners Healthcare.

TABLE 1 | Demographic data and HIV-associated parameters.

	HIV+/ATB	HIV+/LTB	HIV-/ATB	HIV-/LTB
Total number	12	22	21	22
Mean age (years ± SD)	34,8 ± 7.6	32,8 ± 7.4	40,2 ± 10.5	28,6 ± 8.6
Gender (Females)	6 (50%)	17 (77%)	8 (38%)	9 (41%)
Viral load mean (copies/ml ± SD)	127722,5 ± 280219,9	38225,3 ± 62464,65		
CD4+ T cell count mean (cells/mm^3 ± SD)	206,2 ± 183,6	506,8 ± 295,0		

Antigens

Two hundred and nine Mtb antigens were used in this project. Purified LAM was received from BEI Resources and purified protein derivative (PPD) was obtained from the Statens Serum Institute. The remaining 207 proteins were recombinantly expressed Mtb antigens received from Dr. Tom Ottenhoff and Kees Franken (**Table S1**), and prepared as described previously (33, 34). These antigens were selected based on their immunogenicity and their discriminatory potential for TB diagnosis (33, 35, 36).

Luminex Beads Coupling and Antigen-Specific Immunoglobulin Quantification

Antigen-specific antibody subclass and isotype levels present in the plasma of individuals with TB were measured using a custom multiplexed Luminex assay, as previously described (37). This customized Luminex platform has been used extensively across diseases (38–41). Mtb antigens were coupled to magnetic carboxylated fluorescent Luminex beads (Luminex Corporation) by carbodiimide-NHS ester coupling, with one bead region per antigen. Before the coupling with the antigens, beads were activated in an activation buffer containing 100 mM monobasic sodium phosphate (pH 6.2), in addition to 50 mg/ml N-hydroxysulfosuccinimide (Sulfo-NHS; Pierce) resuspended in H_20 and 1-ethyl-3-[3-dimethlyaminopropyl]carbodiimide-HCl (EDC; Pierce) resuspended in activation buffer. After a 30-minute incubation at room temperature (RT), beads were washed in coupling buffer (50 mM morpholineethanesulfonic acid (MES; pH 5.0)), then incubated with Mtb antigens for 2 hours at RT. Beads were then blocked during a 30 minute incubation at RT in phosphate buffered saline (PBS)-TBN (0.1% bovine serum albumin [BSA], 0.02% Tween 20, and 0.05% azide [pH 7.4]). Finally, beads coupled to proteins were washed in PBS-Tween (0.05% Tween 20) and stored in PBS with 0.05% sodium azide at 4°C. As LAM is a glycolipid, a modification by COOH-4-(4,6- dimethoxy[1,3,5]triazin-2-yl)-4-methyl-morpholinium (DMTMM) was required before the coupling to Luminex beads, as described previously (42). For this protocol, 2 µl of DMTMM (200 mg/ml; Sigma-Aldrich) were used for 25 µg of LAM. After 1 hour of incubation at RT, the excess of DMTMM was removed by using Sephadex G-25 PD-10 desalting columns (GE Healthcare). LAM was then added to the beads and incubated overnight at RT. The next day, LAM-coupled beads were washed in PBS then stored in PBS with 0.05% sodium azide at 4°C.

Antigen-coupled beads were added to plasma samples from TB individuals at 1:100 dilution in PBS and incubated at 4°C for 18 hours of shaking. Beads were then washed 3 times with PBS-Tween (0.05% Tween 20) and incubated with phycoerythrin (PE)-conjugated mouse anti-human IgG1, IgG3, IgA1 or IgM (Southern Biotech) at 1.3 µg/ml. After 1 hour of incubation at RT with shaking at 800 rpm, beads were washed 3 times with PBS-Tween (0.05% Tween 20) and resuspended in sheath fluid (Luminex Corporation). Each plasma sample was tested in duplicate, and PE median fluorescence intensity (MFI) levels were measured *via* the iQue screener plus (Intellicyt) analyzer.

Antigen-Specific Fcγ-Receptor Binding

Fcγ-receptors (FcγRs) were purchased from Duke Human Vaccine Institute to study the relative binding levels of Mtb-specific antibodies to individual FcγRs (43). Avi-tagged FcγR2AR, FcγR2B, FcγR3AV and FcγR3B were biotinylated with a BirA biotin-protein ligase (BirA500; Avidity) and the excess of biotin was removed with Zeba spin desalting columns (7K MWCO; Thermo Fisher Scientific).

Diluted plasma samples (1:100) were added to antigen-coupled beads to form immune complexes as described above, and after 18 hours of incubation at 4°C, beads were washed 3 times with PBS-Tween (0.05% Tween 20). At the end of the incubation, streptavidin-R-phycoerythrin (ProZyme) was added to each biotinylated FcγRs in a 4:1 molar ratio during an incubation time of 20 minutes at RT. Fluorescent labeled FcγRs (1 µg/ml in 0.1% BSA-PBS) were then added to immune complexes and incubated for 1 hour at RT. Finally, beads were washed 3 times with PBS-Tween (0.05% Tween 20), then resuspended in sheath fluid (Luminex Corporation) and the median PE intensity was measured *via* the iQue screener plus (Intellicyt) system. Samples were tested in duplicate.

Statistical and Computational Analysis

Data analysis was performed using R version 4.0.2 (2020-06-22). Comparisons between active and latent individuals were performed using a Mann-Whitney U-test test followed by a Benjamini-Hochberg (BH) correction for multiple comparisons.

A multivariate approach, combining a least absolute shrinkage and selection operator (LASSO) for feature selection and classification using partial least square discriminant analysis (PLS-DA) with the LASSO-selected features was used to define feature that discriminated active and latent antibody profiles. Prior to analysis, mean fluorescence (MFI) values were first log-transformed and all data were normalized using z-scoring. Models were built using the R package "ropls" version 1.20.0 (44) and "glmnet" version 4.0.2. Model accuracy was assessed using five-fold cross-validation. For each test fold, LASSO-based feature selection was repeated 100 times, features selected at least 90 times out of 100 were identified as selected features. A PLS-DA classifier was applied to the training set using the selected features, and a prediction accuracy was recorded. The model was also validated *via* permutation testing, where the performance of the selected model was evaluated against randomly shuffled active-latent labels. Selected features were ordered according to their Variable Importance in Projection (VIP) score, and the first two latent variables (LVs) of the PLS-DA model were used to visualize the samples.

RESULTS

A Limited Mtb Antigen Panel Highlights Differences Across ATB and LTB but Does Not Fully Discriminate Across Disease Status

To explore humoral responses in individuals with LTB and ATB, we began by profiling isotype and subclass levels across 8

common Mtb antigens (LAM, PPD, ESAT6/CFP10, Ag85A and 85B, groES and PSTS3) in ATB and LTB individuals that were HIV positive or negative. As previously described, HIV negative ATB individuals exhibited higher levels of IgG1, IgG3 and IgA1 responses to particular antigens, including LAM, PPD, and groES (**Figures 1A, B**). Conversely, an opposite profile was observed in HIV positive individuals, marked by significantly elevated humoral immune responses, and particularly IgM responses to Ag85A and PSTS3 among HIV positive LTB (**Figures 1A, C**). However, these features did not result in complete discrimination of ATB and LTB patients across HIV serostatus (**Figures 1D, E**) (cross-validation accuracy of 62.8% for HIV-; 67.6% for HIV+), suggesting that common antigen-specific antibody profiles provide only a modest level of discrimination across TB infection and disease states.

An Expanded Mtb Antigen Panel Identifies Unappreciated Mtb-Specific Antibody Differences Between ATB and LTB Across HIV Infection Status

Despite the lack of differences across a limited number of common Mtb antigens (**Figure 1**), Mtb can express up to 4000 distinct antigens (45, 46), many of which may serve as additional antibody targets. Thus, we next elected to deeply profile the humoral immune response across LAM, PPD as well as 207 Mtb antigens (**Table S1**), transcriptionally enriched in *in vitro* or *in vivo* infection models, including lung specimens (36). Specifically, IgG1, IgG3, IgA1 and IgM levels were profiled across all antigens in ATB and LTB among HIV positive and HIV negative individuals (**Figure 2**). The data highlighted generally expanded IgG1 and IgG3 responses across HIV negative and positive ATB compared to LTB, with some sporadic elevated IgG1/IgG3 responses in HIV-positive LTB (**Figure 2A**). IgA responses were significantly elevated in HIV negative ATB compared to LTB, despite expanded IgA immunity in HIV-positive LTB (**Figure 2A**). IgM responses were more diffuse across HIV negative groups, despite the presence of a few high IgM responses in ATB compared to HIV negative LTB. Conversely, the IgM response was distinct in the setting of HIV co-infection, marked by significant expansions of particular Mtb-specific IgM responses that were either enriched in ATB or were enriched in LTB (**Figure 2A**). Thus, using this expanded antigen set, striking differences were noted in the evolution of the humoral immune response across HIV-serostatus, pointing to the presence of particular antigen-specific humoral immune responses that may discriminate between TB disease states.

Minimal Sets of Mtb-Specific Antibody Responses Discriminate Between ATB and LTB Across HIV Status

To define the minimal set of specific antigens that were differentially targeted across the groups, we began by quantifying the number of antigens that were differentially targeted by each subclass or isotype of antibodies (**Table S2**).

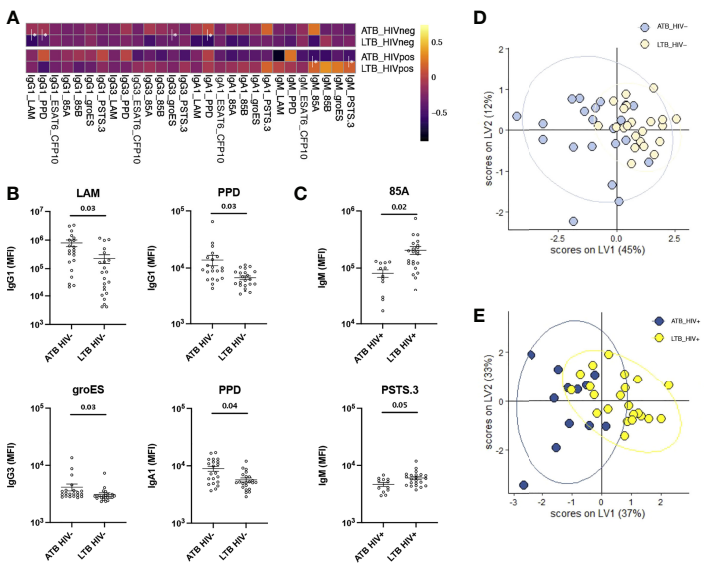

FIGURE 1 | Antibody levels against common Mtb antigens differ between ATB and LTB patients. Relative levels of IgG1, IgG3, IgA1 and IgM against 8 common *Mtb* antigens (LAM, PPD, ESAT6/CFP10, Ag85A and 85B, groES and PSTS3) were quantified *via* Luminex in the plasma of ATB (n=21) and LTB (n=22) patients in the HIV negative group and ATB (n=12) and LTB (n=22) patients in the HIV positive group. **(A)** For the heatmap, Z-score transformation of the MFI values was performed, and the median of each group was graphed. Antibody levels that are significantly different between ATB and LTB are graphed separately for all individuals in the HIV negative **(B)** and the HIV positive **(C)** groups. A Mann-Whitney U-test test followed by a Benjamini-Hochberg (BH) correction for multiple comparisons was used to test for statistically significant differences ATB and LTB. Multivariate analysis using LASSO and PLS-DA model shows antibody levels comparisons between ATB and LTB in HIV negative **(D)** and positive **(E)** populations. Cross-validation accuracy for **(D, E)** was 62.8% and 67.6%, respectively.

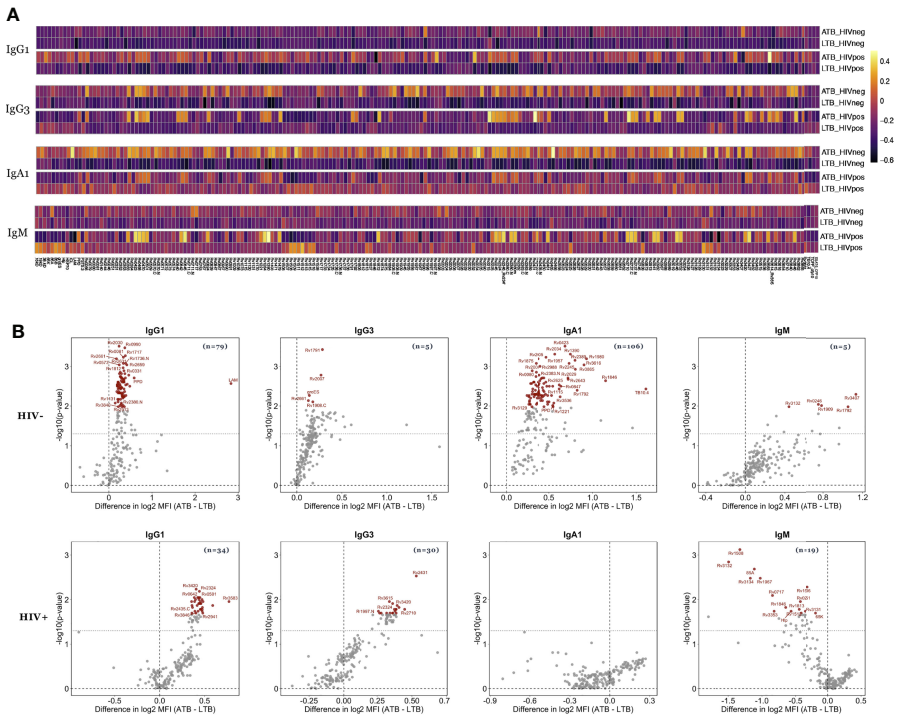

FIGURE 2 | Mtb-specific antibody levels differ between ATB and LTB in HIV negative and HIV positive populations. Relative levels of IgG1, IgG3, IgA1 and IgM against 209 Mtb antigens were quantified *via* Luminex in the plasma of ATB (n=21) and LTB (n=22) patients in the HIV negative group and ATB (n=12) and LTB (n=22) patients in the HIV positive group. **(A)** Heatmaps illustrate the median of Z-scored MFI data for each group indicated. **(B)** The volcano plots characterize the magnitude (log2 fold change of ATB/LTB) and the significance (*p* values) of antibody levels between ATB and LTB. Values above black dashed lines are statistically different between ATB and LTB ($p < 0.05$). For adjusted *p* values, significant data are shown in red, non-significant differences are in black.

Specifically, in HIV negative individuals, 79 Mtb antigens were differentially targeted by IgG1, 5 antigens were differentially targeted by IgG3, 106 were differentially targeted by IgA1, and 5 antigens were targeted distinctly by IgM (**Figure 2B** and **Table S2**). Conversely, in HIV positive individuals, fewer statistically significant differences were observed after multiple correction but included: 34 Mtb antigens that were targeted statistically differently by IgG1, 30 that were differentially targeted by IgG3, and 19 antigens were targeted uniquely by IgM across the two groups (**Figure 2B**). Interestingly, for most comparisons, IgG1, IgG3, and IgA1 levels were higher in ATB individuals, in accordance with published data and likely reflective of the enhanced inflammatory state and bacterial burden observed in ATB infection among HIV positive individuals (22, 23). However, Mtb-specific IgM responses were targeted differently in HIV positive and negative individuals, with higher Mtb-specific IgM responses in ATB in the HIV negative group, but higher Mtb-specific IgM responses in LTB in the HIV positive population, reflecting a shift in B cell response profiles in the setting of HIV. Yet, collectively, these data highlight the striking differences in Mtb-specific humoral immune responses in the setting of HIV infection, but still associated to the persistence of differential humoral immune responses across ATB and LTB.

Differential Mtb-Specific Antibody Binding to Fcγ-Receptors Across Mtb Disease States

Beyond overall changes in antibody subclass and isotype responses to Mtb, emerging data point to significant differences in the inflammatory state of Mtb-specific antibodies across disease states (26, 29). These changes are induced by alterations in antibody-Fc-glycosylation, aimed at deploying antibody effector functions required for enhanced clearance and control of the pathogen (47). Because these Fc-glycosylation changes result in altered binding to Fcγ-receptors (FcR), we next examined whether Mtb-specific FcR binding differences (FcγR2AR, FcγR2B, FcγR3AV, and FcγR3B) existed against LAM, PPD and 207 Mtb antigens (**Table S1**) in HIV positive and negative individuals with ATB or LTB (**Figure 3**).

Thirty-nine Mtb antigens were differentially recognized by FcγR2AR binding antibodies in HIV negative LTB populations compared to ATB (**Figure 3B**). Moreover, 54, 31 and 50 Mtb antigens were differentially recognized by FcγR2B, FcγR3AV and FcγR3B-binding antibodies, respectively, with higher binding observed in ATB (**Figures 3A, B** and **Table S2**). Conversely, FcR binding antibodies were globally shifted in HIV positive individuals, marked by disproportionately higher

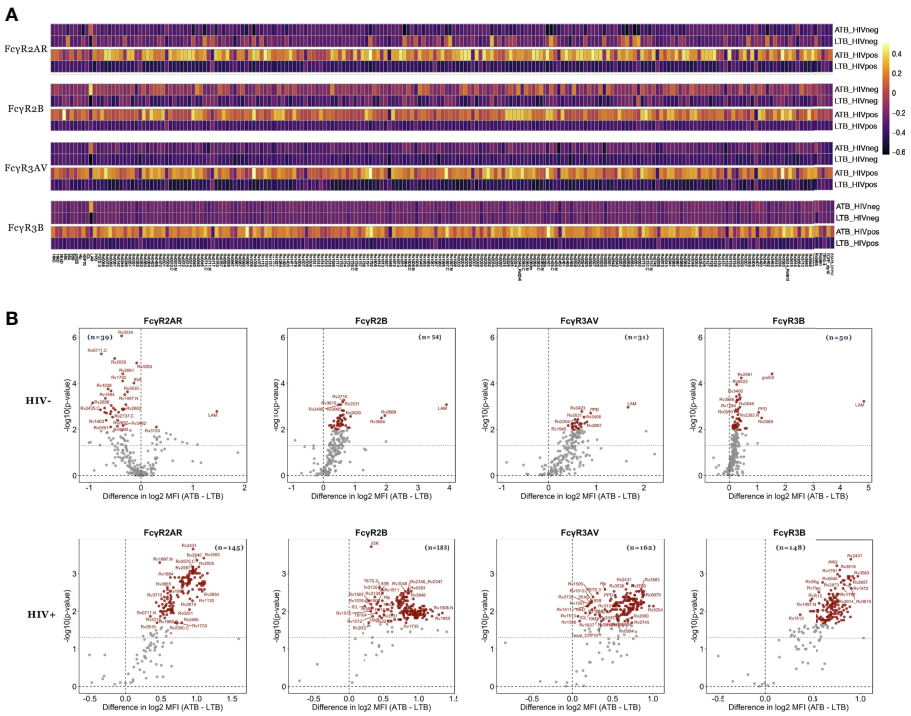

FIGURE 3 | Mtb-specific FcR binding distinguishes ATB from LTB among HIV negative and positive individuals. The ability of antibodies to bind to FcgR2AR, FcgR2B, FcgR3AV and FcgR3B was measured in the plasma of ATB (n=21) and LTB (n=22) patients without HIV infection as well as ATB (n=12) and LTB (n=22) patients with HIV. **(A)** Heatmaps show median values of FcR binding after Z-score transformation to each of the 209 Mtb antigens. **(B)** Fold change between FcR binding in ATB and LTB as well as the significance (*p* values) of differences are plotted the volcano plots. Values above black dashed lines are statistically different between ATB and LTB ($p < 0.05$). For adjusted *p* values, significant data are shown in red, non-significant differences are in black.

levels of FcγR2AR, FcγR2B, FcγR3AV and FcγR3B binding antibodies in HIV+ ATB compared to LTB (**Figure 3**). Specifically, 145, 183, 162 and 148-Mtb-antigens were targeted more robustly by FcγR2AR, FcγR2B, FcγR3AV and FcγR3B binding antibodies, respectively, in ATB compared to LTB (**Table S2**). Thus, beyond alterations in overall antibody subclass/isotype binding profiles across TB disease status, alterations are observed with FcR binding, that are amplified in the setting of HIV co-infection, again pointing to the possibility for antibody-based discrimination across LTB and ATB across HIV status.

A Minimal Set of Antibody Biomarkers Can Discriminate LTB and ATB Irrespective of HIV Status

Given the significant differences in isotype, subclass, and FcR binding differences across LTB and ATB in both HIV positive and negative individuals, we next aimed to identify a minimal set of potential candidate antigens that could be used to discriminate between ATB and LTB. A computational analysis that combined LASSO feature down-selection and PLS-DA classification was conducted in the HIV negative (**Figures 4A–D**) and positive (**Figures 4E–H**) groups. Out of the 1680 antigen-specific antibody features included in the analysis, only 4 features were sufficient to separate HIV negative ATB and LTB (**Figures 4A, B**): RV2034-specific FcγR2AR binding, which was higher in LTB, as well as LAM-specific FcγR3A binding antibodies, RV1528-specific FcγR2B binding antibodies, and RV2435c IgG1 levels that were enriched in ATB (**Figure 4B**). Three of the LASSO selected features were significantly differentially targeted across the groups, including RV2034-specific FcγR2AR binding, LAM-FcγR3A binding and RV1528-specific FcγR2B binding antibodies (**Figure 4C**). Given that LASSO selects a minimal set of features that account for the greatest variance across the groups, the LASSO-selected features may represent groups of correlated antibody features that diverge across TB groups. Thus, to gain enhanced insights into the global changes across groups, a co-correlate analysis was built on the LASSO-selected features highlighting the presence of a large, expanded FcγR2B network in ATB, as well as two smaller networks of LAM-biomarkers and FcγR2AR binding biomarkers enriched in HIV negative ATB individuals (**Figure 4D**).

Similar to the HIV negative LTB/ATB individuals, as few as 3 of the 1680 analyzed features were sufficient to separate LTB and ATB individuals with HIV (**Figures 4E, F**). Specifically, RV3583 FcγR2AR binding was enriched in ATB individuals, and RV1508 and Ag85A IgM levels were enriched among LTB (**Figures 4E, F**). All features were significantly different across the groups

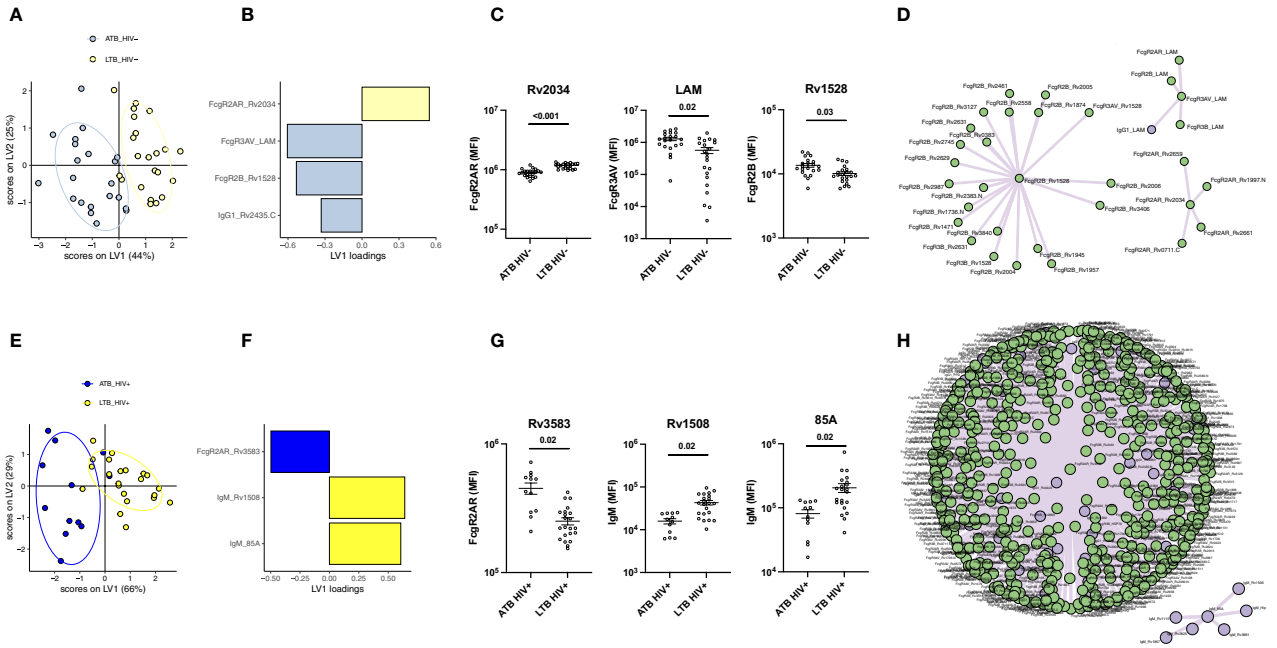

FIGURE 4 | Individuals with ATB and LTB exhibit distinct humoral profile against Mtb antigens regardless of HIV infection. Multivariate analysis comparing antibody response in ATB and LTB among HIV negative **(A, B)** and HIV positive **(E, F)** populations. **(A, E)** PLS-DA models showing dot plots of nearly non-overlapping antibody features in ATB (blue) and LTB (yellow) from HIV negative **(A)** and positive **(E)** groups. Ellipses show 95% confidence intervals. PLDS-DA models were trained using LASSO-selected features that are plotted on VIP plots for HIV negative **(B)** and positive **(F)** patients. Cross-validation accuracy for **(A, E)** was 74.2% and 79.1%, respectively. Antibody features showing significant differences between ATB and LTB are graphed for the HIV negative **(C)** and HIV positive **(G)** cohorts. A Mann-Whitney U-test test followed by a Benjamini-Hochberg (BH) correction for multiple comparisons was used to test for statistically significant differences ATB and LTB. Network correlations depicting the additional non-LASSO-selected features that were correlated with the LASSO-selected parameters statistically different between ATB and LTB in the HIV negative **(D)** and positive **(H)** groups. Data on antibody levels are in purple, features related to FcγR binding are in green.

(**Figure 4G**). Interestingly, co-correlates analysis revealed that the single ATB feature was tightly linked to nearly all IgG/FcR binding levels that were globally expanded in ATB (**Figure 4H**). Additionally, a smaller network of IgM features emerged, marking the unique expansion of IgM responses among HIV positive LTB.

Finally, we aimed to estimate the classification accuracy of the LASSO-selected antibody features across ATB and LTB. An area under the receiver operating characteristic (ROC) curve (AUC) was calculated for each feature (**Figures 5A, B**). Importantly, the combination of the selected antibody features gave an AUC close to 1 (AUC = 0.98 for HIV-; AUC = 0.92 for HIV+). However, even using individual antibody features, classification accuracies of 0.96, 0.8, 0.71 and 0.74 were observed with Rv2034-specific binding to FcγR2AR, LAM-specific binding to FcγR3AV, Rv1528-specific binding to FcγR2B and Rv2435C-specific IgG1 levels, respectively, among HIV negative individuals (**Figure 5A**). Similarly, in HIV positive individuals, individual feature AUCs reached 0.81, 0.86 and 0.84 for Rv1508-specific IgM levels, binding of Rv3583-specific antibodies to FcγR2AR and Ag85A-specific IgM levels, respectively (**Figure 5B**). Our results showed that 4 parameters were systematically enriched in ATB compared to LTB among HIV negative and positive populations, which are IgG1 levels against Rv2435.C, as well as Rv3583-, Rv1528- and LAM-binding to FcγRAR, FcγR2B and FcγR3AV, respectively (**Figure 5C**). Thus, a minimal set of largely novel Mtb-specific humoral biomarkers may provide a unique opportunity to help discriminate between LTB and ATB. Given the ease of generation of rapid point-of-care antibody-based diagnostics, these data point to a simple opportunity to develop rapid point-of-care tests for the diagnosis of Mtb disease state.

DISCUSSION

The lack of rapid and specific point-of care diagnostic tools has impeded the control of tuberculosis, particularly among HIV positive individuals (WHO report 2020; (4, 48, 49). Serum antibody-based tests represent promising alternatives to the current medically intensive diagnostic approaches associated to sputum collection for culture or non-specific tests like TST or IGRA to diagnose active TB. However, previous work with strict quantitative measurements of canonical antigen-specific antibody levels has failed, in the past, to identify Mtb-specific humoral biomarkers able to discriminate ATB and LTB (50). We thus aimed to broaden the scope of biomarker discovery, integrating both qualitative differences in humoral immune profiles as well as the breadth of antigen-specific antibody responses across individuals with/without TB among HIV positive and negative adults. In addition to the 8 common Mtb

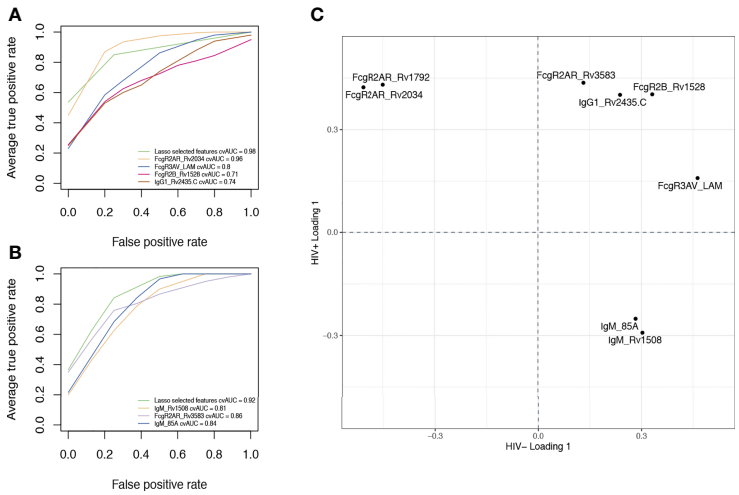

FIGURE 5 | Discriminatory features between ATB and LTB. ROC curves showing the sensitivity and the specificity of potential biomarkers discriminating ATB and LTB in HIV negative **(A)** and positive **(B)** populations. **(C)** Loading plot showing LASSO-selected features enriched in ATB, both among HIV negative and HIV positive individuals (upper right quadrant) versus those enriched in LTB among HIV negative and HIV positive groups (low-left quadrant). Features in the upper left quadrant are increased in ATB compared to LTB among HIV+ but are enriched in LTB among HIV-. Features in the low right quadrant are enriched in LTB compared to ATB among HIV+ but are higher in ATB among HIV-.

antigens (LAM, PPD, ESAT6/CFP10, Ag85A and 85B, groES and PSTS3), 201 additional novel Mtb antigens were included, collectively providing significant resolution of ATB and LTB in HIV negative populations, including higher levels of IgG1 against LAM and PPD in ATB individuals, as well as increased groES-specific IgG3 and PPD-specific IgA1 levels (**Figures 1**, **2**). Conversely, while HIV positive ATB/LTB were also resolvable, the antigen-specific antibody profiles used to discriminate these groups shifted to include higher 85A- and PSTS3-specific IgM response in LTB due to expected HIV induced alterations in immunity. This immune dysregulation in ATB patients with HIV might in part be attributable to lower $CD4^+$ T cell counts and higher viral loads, resulting in altered B cell responses and elevated inflammation (**Table 1**). However, collectively, a set of antigen-specific antibody profiles were defined in both HIV negative and positive populations that provide antigen-specific resolution that could guide TB diagnostic development, providing a unique opportunity to build point-of-care diagnostic tools that would improve TB medical care.

Increased IgG and IgA responses have been previously observed in ATB individuals (22, 24, 30). Studies have shown that high titers are correlated with bacillary load in patient sputum (51). Interestingly, only a weak separation was observed between ATB and LTB when using a limited list of common Mtb antigens (accuracy: 62.8%) (**Figure 1**). Similarly, other studies have shown that antibodies targeting known antigens including Ag85, ESAT6, and CFP10 have limited diagnostic accuracy. Moreover, conflicting reports have demonstrated that the sensitivity of Ag85-specific IgG levels reaches 67.5% for the diagnosis of ATB (50), while others suggested that Ag85-specific IgG levels can provide up to 84.1% (52). Conversely, here the expansion of antigens to additional targets that may be expressed more abundantly in the lung during Mtb infection (36), improved diagnostic accuracy to 98% in HIV negative individuals and 92% in HIV positive subjects (**Figure 5**). Whether these antigen-specific antibodies contribute to the immune response to Mtb remains unclear but may reflect the presence of a more robust host (antibody) response to highly expressed antigens, that may more sensitively resolve individuals with differential control of Mtb.

Surprisingly, HIV co-infection abrogated the differences in IgG and IgA observed in ATB compared to LTB among HIV negative individuals. In accordance with our observations, data published previously reported that total amounts of LAM IgG were higher in ATB/HIV- compared to ATB/HIV+ individuals (30). Also, the loss of IgM observed in ATB individuals with HIV points to a significant shift in the humoral immune response upon co-infection (53). Moreover, given our emerging appreciation for the importance of IgM in Mtb control (54), it is plausible that the loss of IgM may compromise anti-microbial control in HIV/Mtb co-infection, contributing to disease progression. IgM is known for its antimicrobial function, inducing opsonophagocytosis, complement deposition and agglutination (55). Studies in mice have highlighted the main role that IgM plays in the control of Mtb infection by regulating immune response inside granulomas, leading to improved survival of infected mice during the chronic phase of the disease (56, 57). Here we observed that HIV positive individuals with LTB had higher IgM levels against particular Mtb antigens compared to individuals with ATB, including responses against Ag85A and PSTS3, potentially pointing to specific IgM targets that may represent critical Mtb therapeutic targets (**Figures 1**, **2**).

In addition to isotype differences across ATB/LTB, Mtb-specific FcR binding profiles were significantly differentiated across the groups, both in HIV positive and negative populations (**Figure 3**).

Changes in FcR binding are attributable to both alterations in subclass distribution and Fc-glycosylation (58, 59), both of which shift rapidly in response to inflammatory cues that arise during infection. Thus, FcRs may act as sensitive markers of inflammatory changes in antibodies (47). Similarly, previous studies have highlighted striking differences in Fc-glycosylation across HIV negative LTB and ATB (26, 29). Moreover, despite the robust general Fc-inflammatory changes associated with HIV (60, 61), Fc-glycosylation changes still likely occur in the setting of HIV co-infection, marked by striking, and previously unappreciated highly significant increases in all FcR binding in the setting of ATB infection across most Mtb antigens (**Figure 3**). Interestingly, while the differences are not as pronounced in HIV negative ATB/LTB, Mtb-specific FcR measurements were still key to resolving the groups. FcR binding differences are likely attributed to significant changes in Fc-glycosylation, that can be detected using Mass-spectrometry, but may be more readily quantified, in a simpler point-of-care approach using FcR detection.

Specific antigen-dependent antibody responses were key to discriminating ATB and LTB. In HIV negative individuals, IgG1 levels against Rv2435.C as well as FcγR2AR, FcγR3AV and FcγR2B binding associated with Rv2034, LAM and Rv1528 respectively were sufficient for the separation of the groups (**Figure 4B**). In coinfected individuals, only 3 parameters were enough for ATB versus LTB discrimination: Rv3583-specific binding to FcγR2AR in addition to Rv1508- and Ag85A-specific IgM levels (**Figure 4F**). Other than LAM and Ag85A, the additional targets are less well defined. Rv2034 has been described as a transcriptional regulator form the ArsR family (62) and shown to be expressed during pulmonary infection, inducing a strong T cell response (63), and clearly - now - also a robust antibody response. Little is known about Rv1529 and Rv2435.C, the former thought to be a polyketide-associated acyltransferase involved in lipid metabolism (64), and Rv2435.C has been defined as an adenylyl cyclase essential for Mtb survival (65, 66). In HIV positive individuals, antibodies to Rv3583, a transcriptional regulator (67), were among the most discriminatory Mtb responses. Finally, IgM responses to Ag85A and Rv1508, the latter involved in cell surface structures and transport (68), were highly discriminatory. Interestingly, 4 features were similarly enriched in ATB compared to LTB in both HIV positive and negative groups, including RV2435c IgG1 levels as well as Rv3583-, Rv1528- and LAM-binding antibodies to FcγRAR, FcγR2B and FcγR3AV, respectively (**Figure 5C**). While it remains unclear how BCG-vaccination alters these antibody responses across populations, these data suggest that by including a set of 7 antigens and 3 Fc-readouts in a multiplexed diagnostic test, it may be possible to develop a single set of antigens and Fc-detectors to distinguish LTB and ATB across HIV positive and negative populations.

Collectively, this work identifies a number of novel antibody targets in ATB and LTB that may help guide diagnostic development. Specifically, these data point to novel combinations of Mtb antigen specificities and antibody Fc-qualities in discriminating between ATB/LTB. Indeed, in addition to LAM, RV2435c, Rv3583 and Rv1528 were also essential for the discrimination of ATB and LTB regardless of HIV status. While several combinations of inflammatory cytokine or transcriptomic biomarkers have been proposed as new exploratory approaches to help discriminate between ATB and LTB, the development of a simple Mtb-antigen-specific antibody point of care diagnostic would provide simplicity and ease of diagnosis. Moreover, while a single Mtb-antigen antibody Fc feature that could discriminate across ATB/LTB in both HIV negative and positive individuals would be desirable, a single solution may not be possible. However, with the advent of novel, simple lateral flow diagnostics that can multiplex antigen and detectors, the combination of the 7 antigens with unique detectors could be easily developed, providing a simple diagnostic to rapidly determine ATB/LTB status in any particular population. Whether additional antigen-specificities, not probed here, could add additional resolution is possible, and warrants additional analysis. Moreover, further discriminatory antibody profile discovery in IGRA-negative individuals living in TB-endemic areas, among BCG-vaccinated individuals living outside of endemic regions, and analysis of LTB and ATB from disparate geographical regions will provide a better insight into the robustness of these antibody signatures. Additionally, future functional and Fc-glycosylation profiling may reveal whether these unique humoral signatures also track with differential control of the infection, which might lead to the identification of features in LTB individuals that are associated with high risk of progression to ATB. While the ultimate development of a TB diagnostic will require extensive validation across multiple populations globally, overall, our data expand the current knowledge of Mtb antigen-specific humoral immunity and highlights the need to deeply understand how humoral changes may be leveraged both in vaccine and diagnostic development.

DATA AVAILABILITY STATEMENT

The original contributions presented in the study are included in the article/**Supplementary Material**. Further inquiries can be directed to the corresponding author.

ETHICS STATEMENT

The studies involving human participants were reviewed and approved by Human Research Ethics Committee of the University of Cape Town and the Western Cape Department of Health, and the study institutional review board at Massachusetts General Hospital and Partners Healthcare. The patients/participants provided their written informed consent to participate in this study.

AUTHOR CONTRIBUTIONS

GA and NN designed and conceived the research study. NN and KF performed the experiments. DC, EI, WJ, and NN analyzed the data. MK and WH contributed with study cohort

administration and implementation. NN and GA wrote the manuscript, with contributions from DC, LD, BF, KF, CD, and TO. All authors contributed to the article and approved the submitted version.

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SUPPLEMENTARY MATERIAL

The Supplementary Material for this article can be found online at: https://www.frontiersin.org/articles/10.3389/fimmu.2022.856906/full#supplementary-material

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Evaluation of Host Protein Biomarkers by ELISA From Whole Lysed Peripheral Blood for Development of Diagnostic Tests for Active Tuberculosis

*Harriet N. Garlant[1], Kalaiarasan Ellappan[2], Matthew Hewitt[1], Prem Perumal[1], Simon Pekeleke[1], Nadina Wand[1], Jo Southern[3], Saka Vinod Kumar[2], Harish Belgode[2], Ibrahim Abubakar[3], Sanjeev Sinha[4], Seshadri Vasan[5], Noyal Mariya Joseph[2] and Karen E. Kempsell[1]**

[1] Science Group: Research and Evaluation, UK Health Security Agency, Salisbury, United Kingdom, [2] Department of Microbiology, Jawaharlal Institute of Postgraduate Medical Education and Research, Puducherry, India, [3] School of Life & Medical Sciences, Mortimer Market Centre, University College London, London, United Kingdom, [4] Department of Medicine, All India Institute for Medical Sciences, New Delhi, India, [5] Department of Health Sciences, University of York, York, United Kingdom

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Edited by:
Harriet Mayanja-Kizza, Makerere University, Uganda

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***Correspondence:**
Karen E. Kempsell
Karen.Kempsell@phe.gov.uk

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Tuberculosis (TB) remains a significant global health crisis and the number one cause of death for an infectious disease. The health consequences in high-burden countries are significant. Barriers to TB control and eradication are in part caused by difficulties in diagnosis. Improvements in diagnosis are required for organisations like the World Health Organisation (WHO) to meet their ambitious target of reducing the incidence of TB by 50% by the year 2025, which has become hard to reach due to the COVID-19 pandemic. Development of new tests for TB are key priorities of the WHO, as defined in their 2014 report for target product profiles (TPPs). Rapid triage and biomarker-based confirmatory tests would greatly enhance the diagnostic capability for identifying and diagnosing TB-infected individuals. Protein-based test methods e.g. lateral flow devices (LFDs) have a significant advantage over other technologies with regard to assay turnaround time (minutes as opposed to hours) field-ability, ease of use by relatively untrained staff and without the need for supporting laboratory infrastructure. Here we evaluate the diagnostic performance of nine biomarkers from our previously published biomarker qPCR validation study; CALCOCO2, CD274, CD52, GBP1, IFIT3, IFITM3, SAMD9L, SNX10 and TMEM49, as protein targets assayed by ELISA. This preliminary evaluation study was conducted to quantify the level of biomarker protein expression across latent, extra-pulmonary or pulmonary TB groups and negative controls, collected across the UK and India, in whole lysed blood samples (WLB). We also investigated associative correlations between the biomarkers and assessed their suitability for ongoing diagnostic test development, using receiver operating characteristic/area under the curve (ROC) analyses, singly and in panel combinations. The top performing single biomarkers for pulmonary TB versus controls were CALCOCO2, SAMD9L, GBP1, IFITM3, IFIT3 and

SNX10. TMEM49 was also significantly differentially expressed but downregulated in TB groups. CD52 expression was not highly differentially expressed across most of the groups but may provide additional patient stratification information and some limited use for incipient latent TB infection. These show therefore great potential for diagnostic test development either in minimal configuration panels for rapid triage or more complex formulations to capture the diversity of disease presentations.

Keywords: biomarker, protein, ELISA, tuberculosis, diagnostic, assay, diagnosis

1 INTRODUCTION

Tuberculosis (TB) continues to be a leading cause of morbidity and mortality worldwide, accounting for the deaths of an estimated 1.5 million people each year, including 214,000 among HIV positive people in 2020 (1). This figure is comparable to the 1.8 million deaths due to COVID-19 alone during the current pandemic which is also contributing to TB resurgence (2, 3). India has the highest global TB burden, accounting for one fifth of the TB incidence worldwide, with 40% of the total Indian population estimated to be infected with TB (4). In the UK, most TB cases are concentrated in large urban centres where the incidence can be greater than 1/20,000 - one of the highest rates of any Western country (5). Most TB cases (72.8%) occur among non-UK born individuals, who have emigrated from countries with a high burden of endemic TB (6). TB presents predominantly as the pulmonary manifestation (PTB) in the lung, but can affect any organ or tissue, manifesting as a myriad of presentations termed 'extra-pulmonary' tuberculosis [EPTB (7–10)]. This form of the disease is hard to diagnose using conventional methods, as is quiescent or latent TB infection [LTBI (4, 11, 12)]. Therefore, despite ongoing investment in research and development for new diagnostics and therapeutics, TB eradication has proved challenging (13).

Ending the Global TB epidemic by 2030 is a priority in the newly adopted WHO Sustainable Development Goals (14). Rapid non-sputum-based tests for detecting TB and community-based triage tests for identifying suspected TB infected individuals are key priorities for the World Health Organization target product profiles (TPPs), set out in their 2014 report (15). Additionally, a test able to diagnose LTBI or incipient active TB (iATB) would greatly improve early diagnosis, assist with patient management programs, reduce disease dissemination and the current socio-economic disease burden (16–18). There is therefore clearly an urgent need for the development of rapid, inexpensive and accurate tests for diagnosis of TB particularly in the point of care (POC) and remote settings (19–23). A biomarker protein-based, non-sputum diagnostic test such as an LFD would fulfil these criteria. The development of multiplex LFDs able to detect large numbers of analytes simultaneously has recently been achieved (24), however LFDs with two to three analytes are more commonly reported (25). Therefore, in order to configure LFDs for TB triage diagnosis, minimal analyte configurations are most likely required. Multianalyte configurations could be useful in more complex assay formats, e.g. ELISA in the laboratory setting and amenable to confirmatory test application.

Host immune biomarkers which are specifically and differentially expressed during exposure or infection have become an attractive prospect for the detection and diagnosis of TB (11). There have now been a large number of studies conducted to identify and validate high performing, TB disease-specific biomarkers as RNA targets, which have shown potential for use in identifying individuals with exposure or infection with MTB in all its varying presentations, viz. PTB, EPTB and LTBI. Diagnostic and prognostic biomarkers, which are predictive of adequate responsiveness to treatment and of risk of developing active TB in LTBI are also major goals for TB investigators and clinicians (3). However, nucleic acid targets are more amenable to laboratory-based devices and the methodologies have relatively long processing and turnaround times, even if they can be rapidly adapted for new or emerging pathogens, strains and variants (26, 27).

There have been fewer reports of biomarker indicators assayed as proteins for TB, but some studies have been conducted using saliva, serum and plasma samples (28–34). Protein biomarkers are predicted to be most useful target for simple, fast and cost-effective 'point of care' tests (35) required to improve diagnosis rates in resource-limited settings (36, 37). The upfront work required to develop protein assay reagents is significantly more laborious and time consuming than for nucleic acid targets (24). The rewards with respect to cost and assay turnaround time are significantly higher compared to nucleic acid-based methodologies (38–41).

Here we present a preliminary evaluation study to assess the diagnostic performance of nine of our previously identified, published mRNA biomarkers (13) as protein targets; CALCOCO2, CD274, CD52, GBP1, IFIT3, IFITM3, SAMD9L, SNX10 and TMEM49. Expression was assessed using commercial ELISA assays, these were then analysed individually and in combination panels to establish best candidates for future progression in developing protein-based TB diagnostic POC tests. Expression correlation matrix analyses were also conducted to gain an understanding of their inter-target relationships/influences, biological functional significance and potential for TB disease sub-type stratification.

2 METHODOLOGY

2.1 Sample Collection

A total of 452 peripheral blood samples were collected from three cohorts of TB patients – with pulmonary TB (PTB) or extra-

pulmonary TB (EPTB) collected at two geographically distant areas in Northern and Southern India, at AIIMS, New Delhi (A-EPTB) or JIPMER, Puducherry (J-EPTB); latent TB (LTBI) and control groups (P-CNTRL), collected as part of the PREDICT TB study and two other control groups collected at the partner site AIIMS in New Delhi (A-CNTRL) or in the UK [UK-CNTRL (First Link Ltd., Wolverhampton, UK)]. All TB patients included in the study from India were > 16 years of age. PTB patients were recruited on the basis of sputum Ziehl Neelsen stain (ZN) positivity for acid-fast bacilli (AFB) and eventual culture positivity for TB. EPTB patients were recruited on the basis of ZN positivity for acid-fast bacilli (AFB) and eventual culture positivity for TB, sampled at a body site other than the lung. A description of recruitment and inclusion criteria of individuals to the PREDICT-TB study has been described previously elsewhere (42, 43). Details of individuals from the PREDICT-TB study in the LTBI group, who progressed to active TB disease have been published recently by Gupta et al. (44). Median time to TB disease among the progressors was 188 days (interquartile range, 76–488 d). Controls collected at all sites were > 16 years of age and recruited on the basis on no outward signs of respiratory disease or other infectious disease conditions (asymptomatic). UK-CNTRLs were less than 55 years of age and certified non-reactive to human immunodeficiency (HIV), Hepatitis B and C by approved antigen or antibody enzyme-linked immunosorbent assay (ELISA) methods. The UK-CNTRL and the A-CNTRL control group were not tested for TB. P-CNTRLs were negative for interferon-γ release assay (IGRA); QuantiFERON® TB Gold In-Tube [(QFT-GIT) QIAGEN GmbH, Hilden, Germany], T-SPOT®.TB [(T.SPOT) Oxford Immunotec Ltd, Oxford, UK)] and tuberculin skin test (TST) (43). LTBI samples were identified as being variably positive for all three tests. All patient and control sample details are given in detail in **Supplementary Information S1**, **Table S1.1** and summarised in **Table 1** and study details in **Figure 1**. All ethical approvals for the study were in place prior to sample collection, as described previously (13). Blood samples were collected by venepuncture in lithium heparin tubes and stored at -80°C prior to use.

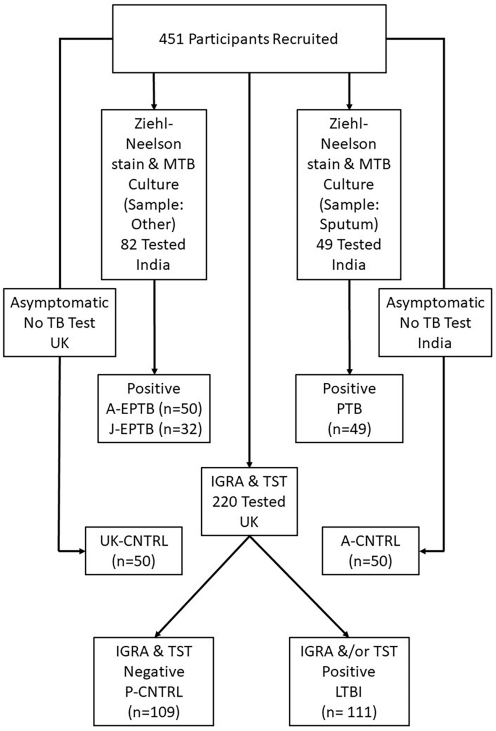

FIGURE 1 | Flowchart of patient and control cohort used in the study.

2.2 Sample Processing

TB infected blood samples were processed at Containment Level 3 and control samples at Containment Level 2 in laboratories at UKHSA, Porton UK or JIPMER, India. In short, whole blood samples were thawed at room temperature, 2 ml aliquots were then transferred to tubes containing 8ml of Invitrogen Cell Extraction Buffer (Life Technologies, UK). These were mixed by inversion and placed on ice for 30 minutes to lyse. All blood lysates were filtered through Millex GP 0.22 um syringe filters, aliquoted and stored at -80°C prior to further use.

2.3 ELISA Assays

Quantification of nine candidate protein biomarkers was conducted using commercially available ELISA assay kits (MyBioSource, USA). The detection range of each assay was as follows; CALCOCO2 (0.78-100ng/ml), CD274 (0.156 -10ng/ml), CD52 (39-5000ng/ml), GBP1 (12.5-1600pg/ml), IFIT3 (0.78-100ng/ml), SAMD9L (1.56-200ng/ml), and TMEM49 (31.2-1000 pg/ml), IFITM3 (3.125-100ng/ml) and SNX10 (6.25-200ng/ml) respectively. All lysed blood samples tested were diluted 1/5 in sample dilution buffer, except for CD52 and GBP1 ELISAs which were tested undiluted. Out of range (high) samples were diluted appropriately in PBS and re-tested.

TABLE 1 | Summary of the number of patients per group for all patient and control samples and affiliations with collaborating site in the study.

Sample Group	Description	Region of origin	Number of samples (n)
UK-CNTRL	UK Negative Controls	First Link Ltd., Wolverhampton, UK (low incidence region)	50
P-CNTRL	PREDICT TB Controls	London, UK (PREDICT TB study)	109
LTBI	PREDICT TB Latent TB	London, UK (PREDICT TB Study)	111
A-CNTRL	AIIMS Negative Controls	AIIMS, New Delhi, India (High incidence region)	50
A-EPTB	AIIMS Extra Pulmonary TB	AIIMS, New Delhi, India	50
J-EPTB	JIPMER Extra Pulmonary TB	JIPMER, Puducherry, India	32
PTB	AIIMS Pulmonary TB	AIIMS, New Delhi, India	49

ELISA results were read using a FLUOStar Omega plate reader (BMG Labtech) at UKHSA Porton Down and a FLUOStar Optima plate reader (BMG Labtech) at JIPMER. Raw data were exported using FLUOStar OMEGA MARS analysis software in Excel format, prior to further analysis (given in **Supplementary Information S1**, **Table S1.2**).

2.4 Data Analysis and Normalisation

Numeric absorbance values from the standard curve, derived from known concentrations of antigen supplied with each ELISA kit, were used to plot 5-parameter logistic curves, from which protein concentrations were interpolated. These were then corrected for dilution factor, as required. Standard curves run on individual plates were cross compared to determine inter-assay variation. Data outputs between plates were normalised using the y-midpoint intersection value, extrapolated from each plate's standard curve. In short, the midpoint was calculated using the highest and lowest data points on the y-axis of each standard curve. A value (χ) was calculated by division of the y-midpoint value of the first assay plate standard curve and the y-midpoint values of each subsequent standard curve respectively. Raw test data output values were then corrected by multiplication using the χ value (given in **Supplementary Table File S1: Table S1.2**).

2.5 Data Analyses

2.5.1 Statistical Analyses

Transformed data values were further analysed using Microsoft Excel for Microsoft 365 MSO (Excel 365), Sigmaplot version 20 (SP20), GraphPad PRISM version 9.0 (GPP9), the statistics software package 'R' x 64 3.4.0 Software ('R'), or the bioinformatics software package GeneSpring™ 14.9 ((GX14.9) Agilent). Summary statistics analyses and boxplot graphical outputs depicting median, min/max and interquartile ranges were generated using SP20. T-Tests between paired groups (two-tailed with unequal variance) and graphical outputs depicting average values and standard error were conducted using Excel 365. Correlation coefficients were calculated using the correlation matrix from multiple variable analyses function (non-parametric spearman correlation, 2-tailed) using GPP9. Normalised data were imported into GX14.9 with no further modification for multivariate analysis of variance (MANOVA), using Benjamini [Hochberg false discovery rate correction (BH FDR $p \leq 0.05$)], T Test (unpaired, unequal variance, no FDR correction) and fold-change analyses.

2.5.2 Random Forest Modelling and ROC Curve Analyses

The performance of each candidate biomarker for discriminating between control and TB disease groups, was determined according to ROC 'area under the curve' (AUC) values, calculated using the 'ROCR' package function in 'R' and the ROC analysis tool of SP20. Cut-off values were predicted by measuring the optimal accuracy of the curve, from which sensitivity and specificity values were calculated. Identification of best performing biomarker panel combinations were predicted by Random Forest modelling using the randomForest package in 'R'. Models were performed to classify all controls from Active TB (classifying PTB and EPTB as separate groups) and all controls from PTB. Data were split (75% training set, 25% testing set), with samples missing data excluded from the analysis. For biomarker combination selections, variables were ranked on decrease in Gini scores. Composite panel scores were calculated from simple additive algorithms consisting of panels of best performing biomarkers from which composite ROC curve analyses were performed to determine optimal best performing panels of biomarkers. Diagnostic performance of these algorithms was also assessed using Sensitivity, Specificity, Cut-off values and Likelihood ratios.

3 RESULTS

3.1 Evaluation of Individual Protein Biomarker ELISA Data

Individual protein biomarker expression within each control and TB disease group was analysed using the summary statistics function in SP20 (**Supplementary Information File S1**, **Table S1.3**; UK-CNTRL, **Table S1.4**; P-CNTRL, **Table S1.5**; A-CNTRL, **Table S1.6**; A-EPTB, **Table S1.7**; J-EPTB, **Table S1.8**; LTBI, **Table S1.9**; PTB). The performance of the assays was generally good, although there are some missing replicates from assay failures. Assay Coefficients of Variability (%CVs) were generally low, although they varied with respect to the biomarker targets and groups, which may reflect either innate or disease-associated expression variation between group individuals.

3.2 Statistical Analysis of Individual Protein Biomarker Expression Profiles

Median, min max and interquartile range expression values are given in boxplot format in **Figure 1** (with a summary of the numeric values given in **Supplementary Information Table File S1** and **Table S1.10** and mean/standard error graphical depictions given in **Supplementary Information Figure File S1** and **Figures S1.1, S1.2**). Good protein expression was observed for most of the biomarker targets, with increased expression for most of the targets in the active TB groups except for TMEM49 and CD52 (which were generally lover in the TB disease groups compared with controls). The A-CNTRL group exhibited higher expression of most of the protein targets assayed than the other control groups. MANOVA analysis across all biomarker targets and groups determined that all nine biomarkers SAMD9L, CALCOCO2, GBP1, IFITM3, SNX10, IFIT3, CD52, CD274 and TMEM49 in the study were significantly differentially-expressed across the groups (**Table 2**). Fold-change analysis using the A-CNTRL group as baseline, revealed the relative differences in expression between this and the other groups. This group was selected as comparator since this is the most likely group reflecting a baseline level of expression against which patients in regions of high endemic TB would be assessed. This analysis also highlighted differences between this control group and the UK-CNTRL, P-CNTRL and LTBI groups, with lower expression of most biomarkers in these groups compared with the A-CNTRL group, except for

TABLE 2 | p-values from MANOVA analysis and fold-change data (using the A-CNTRL group as baseline control) across all biomarkers and groups ■ fold-change ≥ 2.5, ■ fold-change ≥ 1.5, ■ fold-change (white text) ≤ -1.5, ■ fold-change (white text) ≤ -2.5.

Protein Target	Description	MANOVA corrected (BH FDR) p value All Groups	MANOVA uncorrected p value All Groups	PTB vs A-CNTRL Fold Change	J-EPTB vs A-CNTRL Fold Change	A-EPTB vs A-CNTRL Fold Change	LTBI vs A-CNTRL Fold Change	UK CNTRL vs A-CNTRL Fold Change	P-CNTRL vs A-CNTRL Fold Change
CALCOCO2	calcium binding and coiled-coil domain 2	0.00E+00	0.00E+00	**3.53**	1.20	1.17	-1.42	-1.38	-1.66
SAMD9L	sterile alpha motif domain containing 9 like	0.00E+00	0.00E+00	**3.22**	1.72	1.08	-2.31	-1.36	-2.13
IFITM3	interferon induced transmembrane protein 3	0.00E+00	0.00E+00	**2.65**	1.31	1.14	-1.89	-1.47	-1.90
IFIT3	interferon induced protein with tetratricopeptide repeats 3	0.00E+00	0.00E+00	1.97	**2.94**	2.19	**-9.85**	**-9.19**	**-16.82**
GBP1	guanylate binding protein 1	3.13E-39	2.09E-39	1.92	-1.22	1.20	-1.92	-1.51	-1.90
SNX10	sorting nexin 10	4.16E-37	3.23E-37	1.69	1.29	-1.03	-1.59	1.07	-1.68
CD274	CD274 molecule	2.81E-42	1.56E-42	1.63	-1.11	-1.01	-1.19	-1.19	-1.19
TMEM49	VMP1 vacuole membrane protein 1	1.09E-16	9.68E-17	1.25	1.83	-1.32	1.45	1.97	1.53
CD52	CD52 molecule	3.09E-02	3.09E-02	-1.49	-1.24	-1.56	-1.64	-1.52	-1.50

TMEM49. Pairwise statistical analysis between groups confirmed these expression differences (**Supplementary Information File S1** and **Table S1.11**), with varied expression observed across groups (**Figure 1** and **Supplementary Information File S1** and **Table S1.11**).

Significant up-regulation of most biomarkers was observed for the active disease groups, when compared with the UK-CNTRL and P-CNTRL groups, but this was somewhat reduced in comparison with the A-CNTRL group. High average expression of SAMD9L, CALCOCO2, GBP1, IFITM3 and SNX10 was observed in the PTB group compared to the EPTB and other groups, in contrast IFIT3 expression was higher in the EPTB groups. There were significant differences in expression between the two EPTB groups, for SAMD9L, GBP1, SNX10 and TMEM49 expression. Average CD52 expression was higher in the UK-CNTRL and A-CNTRL controls compared with active disease groups. Overall protein expression was significantly lower in the LTBI than all control and Active TB groups for all targets. LTBI as a combined group exhibited significant differences from the UK-CNTRL control group for SAMD9L, GBP1, IFITM3 and SNX10, for IFIT3 with its equivalent control group P-CNTRL and all biomarkers with the A-CNTRL group (**Supplementary Information File S1** and **Table S1.11**).

During study follow up, several individuals (8/106) from the LTBI group were found to have progressed to active disease (LTBI_PR), consistent with the 5-10% lifetime risk of reactivation of TB for an individual with documented LTBI, with the majority developing TB disease within the first five years after initial infection (45). These were separated from LTBI non-progressors (LTBI_NPR) and analysed as a discrete group. T tests revealed expression differences between the LTBI_PR group, the LTBI_NPR and matched P-CNTRL groups for IFITM3, IFIT3 and CD52 (**Supplementary Information File S1** and **Table S1.12**), particularly for CD52 which was approximately two-fold lower in expression in the LTBI_PR group compared with both the LTBI_NPR and P-CNTRL groups (**Supplementary Information File S2** and **Figure S2.2**). A near significant expression difference was observed for CD274 between the P-CNTRL and the LTBI_PR groups.

3.3 Correlation of Biomarker Expression With QuantiFERON, T-SPOT.TB and Tuberculin Skin Test in PREDICT TB Study Samples

Normalised protein target expression data were imported without further modification into the bioinformatics software GX14.9. The P-CNTRL and LTBI groups were annotated with regard to their QFT-GIT, T.SPOT and TST status. Individual T Test analyses (uncorrected, no FDR) were conducted between the two groups for differential biomarker target expression correlating with QFT-GIT, T.SPOT or TST status (significance p values are given in **Supplementary Information File S1** and **Table S1.13**). MANOVA analyses could not be conducted between LTBI_PR and LTBI_NPR groups due to significant variations in group size. QFT-GIT positivity correlated with GBP1 (p-value 0.0196) and TMEM49 (p-value 0.0356)

expression and to a lesser degree of significance with IFIT3 (p-value 0.0544) and SNX10 (p-value 0.079). T.SPOT positivity showed a weak correlation with IFIT3 (p-value 0.095) and TST with CALCOCO2 (p-value 0.0754).

3.4 Correlation Matrix Analysis of Protein Biomarker Inter-Relationships

Correlation coefficients were calculated using the 'correlation matrix from multiple variable analyses function' (non-parametric 2 tailed, Spearman correlation) using GPP9. These are given in heatmap image format in **Figure 2** and corresponding correlation and significance p values in **Supplementary Information File S1** and **Table S1.14**. These showed complex patterns of interactions, varying across the control and disease groups. However, some consistent patterns were observed, with strong correlations between IFITM3 and SAMD9L, CALCOCO2 and SNX10 in all control groups, with reduced positive interaction between IFITM3 and SNX10 in the LTBI, J-EPTB and PTB groups. IFITM3 positive interactions with SAMD9L, CALCOCO2, GBP1 and SNX10 were very reduced in the PTB group. There were also increasingly negative correlations between GBP1 and SNX10, IFIT3 and TMEM49 in the A-EPTB, J-EPTB and PTB groups, GBP1 and CD274 in the E-EPTB group and GBP1 and CD52 in the J-EPTB and PTB groups. Pronounced correlation expression differences were observed between the LTBI_PR and LTBI_NPR groups, particularly for CD52, which exhibited a strong negative correlation with all other biomarkers except CD274 and itself in the LTBI_PR group. These relationships may correlate with shifting immune profiles in the transition from control to latent and then active TB disease states.

3.5 Accuracy of Individual Biomarkers From Whole Blood for Detection of TB Infection

Paired ROC curve analyses were conducted between groups for individual protein biomarkers to determine the relative accuracy of each candidate for discrimination across infected and uninfected groups (given in full in **Supplementary Information File S2**, **Table S2.2** and summarised in **Table 3**). As well as comparisons between individual groups, comparisons were made between all control groups combined (UK-CNTRL/P-CNTRL/A-CNTRL), all EPTB groups and all Active TB groups (A-EPTB/J-EPTB/PTB). Seven of the nine biomarkers were able to discriminate all Active TB from all control groups with good accuracy (ROC ≥ 0.7). IFIT3, IFITM3 and SAMD9L achieved excellent discrimination for Active TB when compared to individual control groups with AUC values between 0.85-1. CD274 performed well for discriminating Active TB from UK-CNTRL and P-CNTRL but fared less well for Active TB from A-CNTRLs. High number of positives were observed in the A-CNTRL compared with the UK-CNTRL and P-CNTRL groups for most biomarkers, suggesting that this group may be heterogeneous and may contain infected or exposed individuals.

Predicted cut-off values were selected to achieve the best sensitivity and specificity of the individual biomarkers for discriminating infected from non-infected individuals. SAMD9L was observed to be the best performing biomarker for PTB [ROC AUC values; PTB vs UK-CNTRL (0.987), P-CNTRL group (0.997), A-CNTRL (0.911), and combined control groups (0.981)]. IFIT3 consistently achieved the highest AUC values for discrimination of EPTB from control groups (ROC AUC values; EPTB vs UK-CNTRL (0.911), P-CNTRL group (1.00) A-CNTRL (0.843) and combined control groups (0.942). Only CD274 showed any reasonable performance for the LTBI group using the A-CNTRL group as comparator (1.00). There appears to be an inverse correlation with CD52, GBP1, IFIT3, IFITM3, SAMD9L, SNX10 and TMEM49 with the other individual control groups for LTBI.

3.6 Improved Performance Combination Biomarker Panels

Single biomarkers were observed to show good performance across all individual groups. However due to the heterogeneity of expression across all TB disease presentations, combined panels using best performing biomarkers were investigated to increase diagnostic performance for all forms of Active TB (including EPTB) and PTB. CALCOCO2, GBP1, IFIT3, IFITM3, SAMD9L and SNX10 were selected for further combinatorial analysis. CD52 and TMEM49 were excluded due to their poor individual ROC curve performances. CD274 was also excluded, as despite its good ROC curve performance for some presentations, its fold-change expression range was very low.

Random forest modelling was performed using the randomForest 'R' package to classify both control and Active TB groups and control and PTB only. Data were randomly split for analysis (75% training and 25% testing), with samples missing data excluded from the analysis. For classification of Controls, EPTB and PTB, the randomForest model showed an Out-Of-Bag (OOB) estimate of error rate of 10.98% with 3 variables tried at each split. SAMD9L, IFITM3 and IFIT3 were ranked highest in importance for the classification of these groups individually [**Figure 4A** (I); **Supplementary Information S2 Table S2.1** (I)]. For classification of PTB from controls only, the randomForest showed an OOB estimate of error 3.11% with SAMD9L, IFITM3 and CALCOCO2 revealed as variables of most importance with 3 variables tried at each split [**Figure 4A** (II); **Supplementary Information S2 Table S2.1** (II)].

Composite panel scores were calculated using simple additive algorithms consisting of the predicted top performing biomarkers to determine in which combination they best discriminated (1) all active TB groups from all control groups and (b) PTB from all control groups (**Supplementary Information File S2** and **Table S2.3**).

One panel combination using a simple additive algorithm of all 6 biomarkers showed superior performance for discrimination of all controls from all active TB;

$$(300 \times IFIT3) \times (3 \times SAMD9L) + GBP1 + IFITM3 + SNX10 + CALCOCO2$$

Figure 5 shows composite box and dotplots of (A) all combined controls vs all combined active TB, (B) all individual

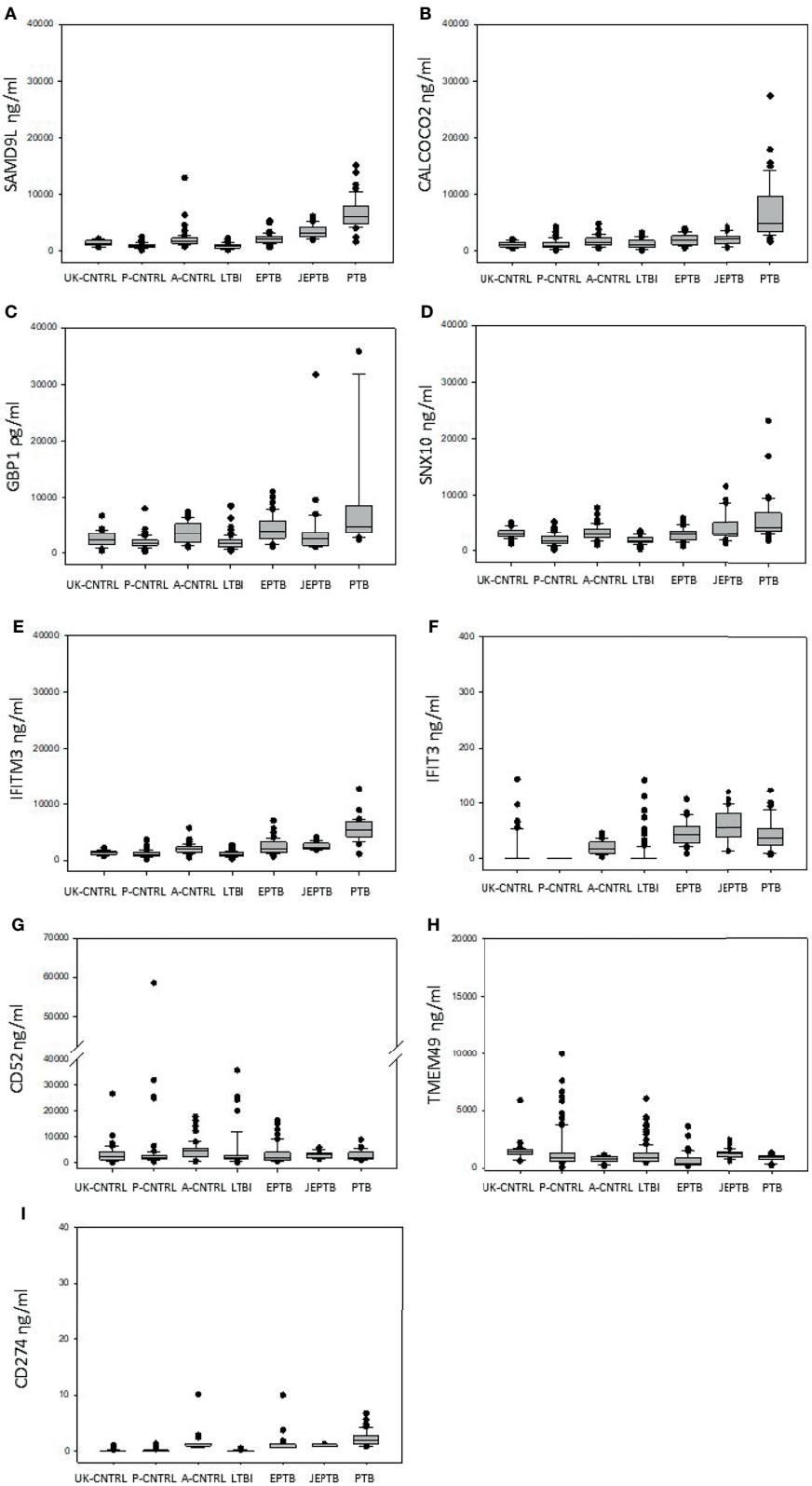

FIGURE 2 | Graphical boxplot depiction of biomarker expression across all control and patient groups (displaying median, minimum, maximum and interquartile expression range) **(A)** SAMD9L **(B)** CALCOCO2 **(C)** GBP1 **(D)** SNX10 **(E)** IFITM3 **(F)** IFIT3 **(G)** CD52 **(H)** TMEM49 **(I)** CD274.

TABLE 3 | Summary of ROC curve values for biomarker expression for all control and TB disease group combinations ROC curve value ≥ 0.9, ROC curve value ≥ 0.8, ROC curve value ≥ 0.7, ROC curve value (white text) ≤ 0.3.

GROUP COMPARISON	CALCOCO2	CD274	CD52	GBP1	IFIT3	IFITM3	SAMD9L	SNX10	TMEM49
UK-CNTRL vs Active TB	0.859	**0.988**	0.520	0.727	**0.907**	0.889	**0.907**	0.611	0.206
UK-CNTRL vs A-EPTB	0.762	**0.978**	0.477	0.764	**0.906**	0.770	0.860	0.444	0.137
UK-CNTRL vs All EPTB	0.781	**0.982**	0.520	0.727	**0.911**	0.850	0.787	0.500	0.241
UK-CNTRL vs J-EPTB	0.809	**0.987**	0.590	0.618	**0.921**	**0.976**	**0.974**	0.590	0.404
UK-CNTRL vs LTBI	0.519	0.534	0.437	0.368	0.492	0.271	0.190	0.138	0.272
UK-CNTRL vs PTB	**0.987**	**0.997**	0.497	**0.900**	**0.901**	**0.953**	**0.987**	0.798	0.148
P-CNTRL vs Active TB	0.853	**0.990**	0.561	0.826	**1.000**	**0.932**	**0.970**	0.838	0.436
P-CNTRL vs A-EPTB	0.767	**0.987**	0.489	0.861	**1.000**	0.870	**0.928**	0.743	0.248
P-CNTRL vs All EPTB	0.779	**0.986**	0.561	0.826	**1.000**	**0.906**	**0.955**	0.780	0.412
P-CNTRL vs J-EPTB	0.797	**0.985**	0.678	0.725	**1.000**	**0.962**	**0.997**	0.841	0.669
P-CNTRL vs LTBI	0.567	0.515	0.452	0.496	0.585	0.504	0.454	0.513	0.484
P-CNTRL vs PTB	**0.976**	**0.997**	0.535	**0.968**	**1.000**	**0.976**	**0.997**	**0.937**	0.476
A-CNTRL vs Active TB	0.736	0.489	0.339	0.537	0.817	0.737	0.793	0.626	0.585
A-CNTRL vs A-EPTB	0.587	0.674	0.337	0.537	0.810	0.568	0.593	0.477	0.363
A-CNTRL vs All EPTB	0.587	0.681	0.339	0.580	0.843	0.622	0.705	0.527	0.554
A-CNTRL vs J-EPTB	0.637	0.690	0.342	0.411	0.897	0.707	0.879	0.608	0.851
A-CNTRL vs LTBI	0.360	**1.000**	0.292	0.224	0.103	0.127	0.087	0.191	0.600
A-CNTRL vs PTB	**0.950**	0.178	0.321	0.727	0.775	**0.930**	**0.941**	0.793	0.637
ALL CNTRLS vs Active TB	0.826	0.875	0.497	0.733	**0.935**	0.874	**0.913**	0.733	0.421
ALL CNTRLS vs A-EPTB	0.723	0.826	0.448	0.770	**0.933**	0.774	0.813	0.608	0.251
ALL CNTRLS vs All EPTB	0.738	0.827	0.497	0.733	**0.942**	0.822	0.872	0.653	0.408
ALL CNTRLS vs J-EPTB	0.762	0.827	0.575	0.624	**0.957**	0.897	**0.963**	0.725	0.654
ALL CNTRLS vs LTBI	0.506	0.396	0.409	0.400	0.449	0.368	0.302	0.346	0.465
ALL CNTRLS vs PTB	**0.972**	**0.955**	0.473	0.893	**0.923**	**0.960**	**0.981**	0.869	0.442
LTBI vs ACTIVE TB	0.711	**1.000**	0.583	0.850	**0.932**	**0.937**	**0.934**	0.872	0.448
LTBI vs A-EPTB	0.721	**1.000**	0.523	670.67	**0.929**	0.875	**0.996**	0.770	0.251
LTBI VS J-EPTB	**0.974**	**1.000**	0.690	**0.951**	**0.945**	**0.976**	**0.998**	0.880	0.693
LTBI vs PTB	0.812	**1.000**	0.573	0.845	**0.926**	**0.976**	**0.973**	**0.972**	0.490

control groups vs all individual active TB groups. ROC curve analyses demonstrated the high performance of this combination for PTB (ROC = 0.9885) compared with the A-EPTB and J-EPTB groups [**Figure 4B** (I)]. Reduced performance was observed for all combined controls vs all combined active TB (ROC = 0.9552).

A refined, simplified panel of these six markers also exemplified all controls from PTB;

CALCOCO2 + *SAMD9L* + *IFITM3*

Figure 6 shows composite box and dotplots of (A) all combined control groups vs PTB, (B) all individual control groups vs PTB. ROC curve analysis demonstrated the high performance of this combination for PTB (ROC = 0.9894) compared with the A-EPTB and J-EPTB groups (**Figure 4B** (II)). Reduced performance was also observed for all combined controls vs all combined active TB (ROC = 0.9079).

Calculated sensitivity, specificity and positive and negative predictive values for the panels were compared with the minimum and optimum technology product profiles for the TB triage test (minimum; 90% Sensitivity/70% Specificity, optimum; 95% Sensitivity/80% Specificity), given in **Supplementary Information File S2** and **Table S2.4** and the TB confirmatory test (minimum; 65% Sensitivity/98% Specificity, optimum; (i) sputum positive PTB 98% Sensitivity/98% Specificity (ii) EPTB 80% Sensitivity/98% Specificity), given in **Supplementary Information File S2** and **Table S2.5**. These results demonstrated that the panels meet the minimum performance criteria for the combined controls vs all active TB groups, with variation in performance observed across the different individual control groups and subtypes of disease. The optimum performance criteria for the triage test was achieved using both panels for many of these latter pairwise comparisons, but the optimum performance criteria was only met for EPTB group comparisons (because of the reduced sensitivity performance level (80%) for EPTB). The minimum requirements were met for the confirmatory test for most of the

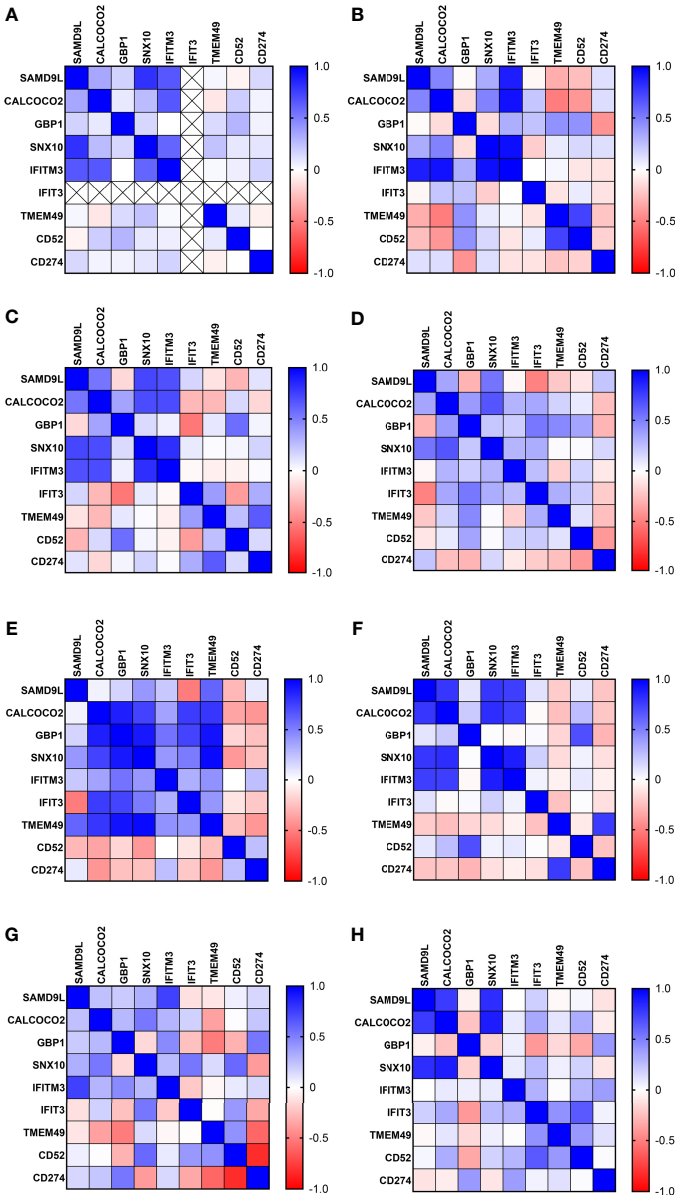

FIGURE 3 | Correlation matrix analysis of protein biomarker inter-relationships in all control and TB disease groups **(A)** UK-CNTRL **(B)** P-CNTRL **(C)** A-CNTRL **(D)** LTBI non-progressors **(E)** LTBI progressors **(F)** J-EPTB **(G)** A-EPTB **(H)** PTB.

pairwise comparisons, but the optimum requirements only for a few using the P-CNTRL or UK-CNTRL groups as comparators e.g. P-CNTRL or UK-CNTRL vs PTB for the simplified panel.

4 DISCUSSION

Here we describe a pilot study to assay select, previously-validated, TB-associated immune mRNA biomarkers CALCOCO2, CD274, CD52, GBP1, IFIT3, IFITM3, SAMD9L, SNX10 and TMEM49 from our previous study (13), as protein targets. These were assayed using commercial ELISA assays at UKHSA, Porton UK and JIPMER, Puducherry, India, using whole lysed blood samples from individuals with suspected LTBI or ATB infection and three groups of controls. Most of the proteins showed expression in the ηg/ml range except GBP1 and TMEM49 which were in the ρg/ml range. Mean and median analyses of the protein biomarkers showed increasing, incremental expression patterns from the P-CNTRL, UK-CNTRL and the A-CNTRL groups through to the EPTB and PTB groups. Higher than expected biomarker expression was also seen in some individuals in the control groups. The A-CNTRL group showed greater number of individuals expressing higher levels of these protein biomarker targets than the other

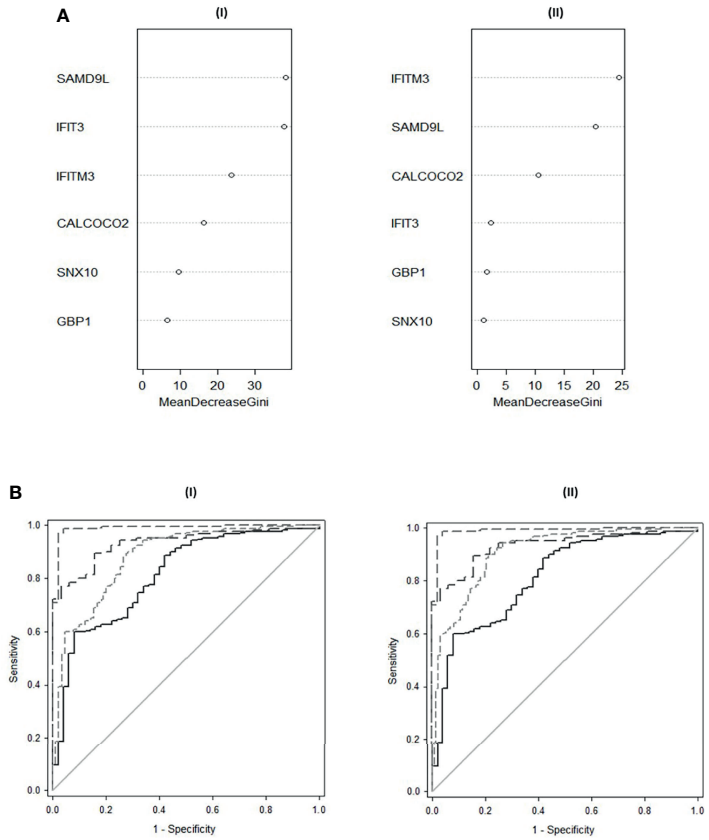

FIGURE 4 | **(A)** Variable Importance Plot of decrease in Gini scores as measured by Random Forest for **(I)** classification of All Controls, EPTB and PTB **(II)** Classification of All Controls and PTB**. (B)** ROC curves of composite panel scores generated from **(I)** the complex 6-plex panel for discrimination of individual TB groups and combined Active TB from All controls **(II)** the simple 3-plex panel for the discrimination of individual TB groups and combined Active TB from All controls. All controls vs A-EPTB ——, all controls vs J-EPTB — —, all controls vs PTB — — —, all controls vs all combined active TB — — — —.

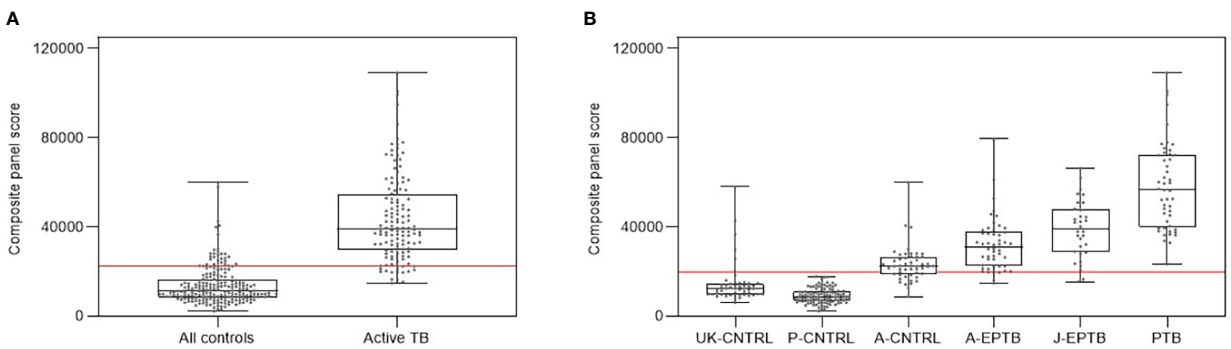

FIGURE 5 | **(A)** Combined box and scatter plot graphical depictions of composite panel score of the complex 6-plex biomarker panel between all controls and all active TB groups combined, displaying the cut-off value y=22361 for discrimination of all active TB groups from all controls with 90.1% sensitivity and 85.7% specificity **(B)** Combined box and scatter plot graphical depictions of expression of the complex 6-plex biomarker panel between individual control and active TB groups displaying the cut-off value y=19698 for discrimination of all active TB groups from all controls with 95.4% sensitivity and 81.3% specificity.

two control groups. Expression in the P-CNTRL and LTBI groups were low for most of the biomarkers and they were highly similar to one another. Few differences were seen between these two groups except with expression of IFIT3, for which there was no recorded expression in the P-CNTRL group, in the dynamic range of the assay. IFIT3 expression in the combined LTBI group exhibited weak statistical correlation with T.SPOT and QFT-GIT but not TST positivity. GBP1 and TMEM49

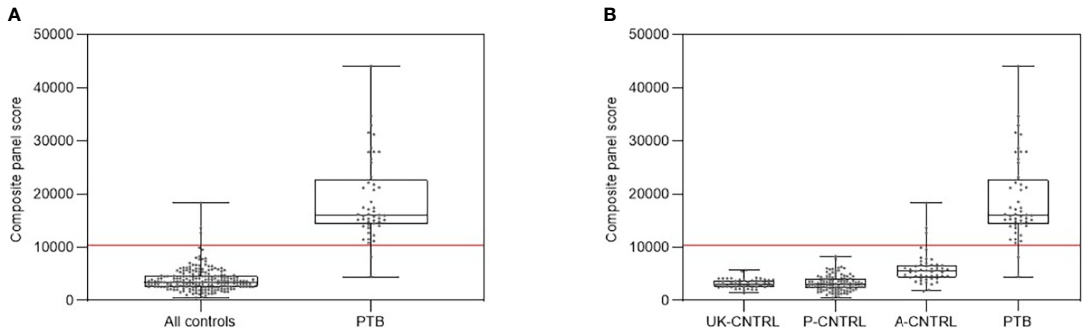

FIGURE 6 | **(A)** Combined box and scatter plot graphical depictions of composite panel score of the simple 3-plex biomarker panel between all controls and PTB, displaying the cut-off value y= 10389 for discrimination of PTB from all controls with 95.9% sensitivity and 98.6% specificity. **(B)** Combined box and scatter plot graphical depictions of expression of the simple 3-plex biomarker panel between individual control and PTB displaying the cut-off value y= 10389 for discrimination of PTB from all controls with 95.9% sensitivity and 98.6% specificity.

exhibited significant correlation with QFT-GIT positivity and to a lesser extent SNX10. Expression of IFIT3 appears to correlate with the LTBI_NPR group and not the LTBI_PR group. CALCOCO2 appeared to show weak correlation with TST. These may reflect diversity in the functionality between the assays and this is reflected in the positivity profiles within the LTBI group (**Supplementary Information S1** and **Table S1.1**). Abubakar et al. noted that combination strategies including all three tests were significantly superior predictors of progression in LTBI patients (43), implying that the use of individual tests gave mixed results. They concluded that multi-testing strategies showed superior performance. The results presented here may imply that the three different tests may demonstrate immunological bias.

Expression levels of SAMD9L CALCOCO2, GBP1, SNX10, IFITM3 and IFIT3 were good and correlated well with TB disease. TMEM49 and CD274 showed more modest expression and appeared more variable in their expression profiles. IFIT3 expression levels were generally low but showed a high degree of specificity to TB disease groups and also some expression in individuals in the A-CNTRL group. As these control samples were collected from a high incidence region of TB infection where the carriage rate of infection is expected to be ≥ 40%, it is postulated that this group could be a heterogeneous population containing a proportion of previously-unidentified TB-exposed or infected individuals. This may highlight issues in discrimination of uninfected and infected individuals from high TB burden regions. However, individuals with increased biomarker abundance above calculated thresholds would be likely candidates for follow-up for suspected TB infection, even if asymptomatic.

Expression differences between the groups were confirmed using MANOVA and pairwise T-tests for nearly all of the biomarkers except CD52, which showed differences between the A-CNTRL, J-EPTB and PTB groups only. Overall, SAMD9L, CALCOCO2, GBP1, IFITM3, IFIT3 and SNX10 showed the most significant expression increase in all the ATB compared to all control groups, with very high expression in the PTB group. Expression differences were observed between the two EPTB groups for SAMD9L, GBP1, SNX10 and TMEM49, suggesting regional variations between these groups. The A-EPTB group showed higher expression of CALCOCO2, GBP1 and IFITM3 than the J-EPTB group, which in contrast exhibited higher expression of SAMD9L, SNX10, IFIT3 and TMEM49. CD274 expression was similar in both groups. CALCOCO2, IFITM3 and CD274 show the most significant expression differences between the PTB group and the two EPTB groups. IFIT3 is elevated in all three disease groups, but is slightly higher in the EPTB groups. These former three biomarkers may be correlated with disease progression and/or severity and could be used to discriminate between these two subtypes of disease, in conjunction with IFIT3 which is expressed in all subtypes of TB. We were able to distinguish LTBI from active TB and high Incidence region controls using most biomarkers but were unable to discriminate LTBI from the matched P-CNTRL control group. Differences in expression for CD52 were seen between the LTBI non-progressors and progressors, this biomarker was significantly downregulated in the latter. These results showed promised for further analysis to determine best performing characteristics or diagnostic purposes.

Correlation coefficient analyses showed somewhat complex patterns of interactions; however, several features were noteworthy. There were strong positive correlations between SAMD9L, CALCOCO2, SNX10 and IFITM3 in all the control groups. These interactions appear lessened in the LTBI and J-EPTB groups and the interaction between IFITM3 and these biomarkers s is severely reduced in PTB. GBP1 and TMEM49 show increasing negative interactions in all three disease groups, A-EPTB, J-EPTB and PTB. There was also a strong negative interaction between GBP1 and CD52 in the J-EPTB and PTB groups. This implies that increasing expression of GBP1 may correlate with disease progression and may be negatively influencing CD52 and TMEM49 expression which appears to be interconnected. CD52 expression was down two-fold in the LTBI_PR group and this appeared to have a negative effect on all other biomarkers except CD274. This implies functional impairment or loss of T-cells in the periphery at an early stage of incipient TB progression by mechanisms unknown, but which

could include trafficking of antigen-specific T-cells to an emerging site of active infection, inhibition or networked cell death (46, 47). All of these proteins are involved in macro-autophagy, particle uptake, trafficking and digestion through phago-lysosomal pathways and are involved in alternate killing of intracellular pathogens.

SAMD9L is a myeloid tumour suppressor gene, which has been shown to involved in the innate response to viral infection (48) and also plays a role in regulating the response to type I interferons in haematopoietic cells and other cell types e.g. T-cells (49). Studies in mice have shown it controls endocytosis of receptors, homotypic fusion of endosomes, and lysosomal activation (50). Other studies have shown SAMD9L to be upregulated in active TB as compared to LTBI infection (51), it may therefore play a role in intracellular uptake and trafficking of TB bacilli. CALCOCO2 plays a similar role in that it functions as a receptor for ubiquitin-coated bacteria and plays an important role in innate immunity by mediating macro-autophagy (52–54). This suggests a role in targeting TB bacilli for degradation by the innate immune system (54–56), which TB has evolved strategies to evade (57, 58). However, increased CALCOCO2 expression may be a double-edged sword as it also targets the signalling adaptor MAVS for ubiquitination and autophagic degradation, hence inhibiting DDX58-mediated type I interferon signalling through a negative feedback loop (59). SNX10 is also involved in intracellular trafficking and may play a role in regulating endosome homeostasis (60, 61). Therefore, these proteins are intimately inter-related functionally and their dysregulation may have consequences for intracellular bacterial trafficking, bactericidal killing mechanisms and have subsequent deleterious downstream effects on interferon-driven adaptive immune responses.

GBP1, IFIT3 and IFITM3 are also interferon-regulated genes and their upregulation in TB disease groups is significant. Both IFIT3 and GBP1 have been implemented in other previously-published TB diagnostic panels (13). Guanylate binding proteins (GBP) like GBP1 are a large family of IFN-induced hydrolases which are necessary for mediation of host innate immune responses (62, 63). GBP1 functions to elicit divergent host cell death programs in response to infection with intracellular pathogens (64), and increases access to PAMPs. IFIT3 functions to inhibit the function of TLR3 (65), which is involved in recognition of cytoplasmic PAMPS like 'foreign' double stranded RNA (66). IFITM3 is a key mediator of the early innate cellular response, however it functions to inhibit phagocytosis, which is beneficial in viral infections, but not with intracellular bacterial pathogens like Listeria which have evolved strategies to exploit its function to avoid phagocyte killing (67). Therefore, upregulation of IFITM3 may inhibit bacterial uptake, but upregulation of GBP1 and parallel downregulation of IFIT3, may lead to GBP-mediated bacterial killing mechanisms and an increase in TLR3-directed immune responses. It is interesting to note the impact of GBP1 on TMEM49 and CD52 and IFITM3 on SAMD9L, CALCOCO2 and SNX10 protein inter-relationships in the PTB group.

SAMD9L, CALCOCO2, SNX10, GBP1, IFIT3, IFITM3 and were all significantly expressed in the active disease groups, making them good candidates for formulation of panels for TB diagnosis. Random Forest modelling and empirical evaluation of many different combinations led to selection of a complex panel, used in combination with the algorithm (300 × *IFIT*3) × (3 × *SAMD9L*) + *GBP*1 + *IFITM*3 + *SNX*10 + *CALCOCO*2), which showed good performance for discrimination of all TB disease groups from all controls (ROC = 0.9552, % sensitivity = 90.43, % specificity = 83.97). This was further evaluated against individual disease groups and demonstrated superior performance for discrimination of the PTB disease group (ROC = 0.9885, % sensitivity = 95.22, % specificity = 97.96, at a cut-off value of 28032) and the J-EPTB group from all controls (ROC = 0.9522, % sensitivity = 95.22, % specificity = 81.25, at a cut-off value of 28032). The panel also met the optimum TPP requirements for all controls vs the combined EPTB groups and the A-EPTB and J-EPTB groups individually, at the reduced requirement for the confirmatory test (80% Sensitivity and 98% Specificity) and all controls vs all combined TB disease and vs the PTB group for the minimum requirements for the confirmatory test (65% Sensitivity and 98% Specificity). The likely platform for this panel is a laboratory-based device, due to its larger, more complex biomarker configuration, however there are groups working on multi-analyte LFD devices which may enable future configuration of this larger panel in LFD format (68–70). It would be useful for diagnosis of smear-positive PTB under the minimum TPP requirements (≥ 65% sensitivity) but not optimum requirements (≥ 98% sensitivity), but for diagnosis of smear-negative PTB (optimal requirements ≥ 68% sensitivity) and EPTB (optimal requirements ≥ 80% sensitivity) at the optimum requirements. We were unable to distinguish a panel to identify LTBI from appropriate demographically-matched control groups.

Ideally a biomarker test should be easy to perform and interpret in a health care setting on point of care devices e.g. lateral flow devices. Evaluation of a reduced, simplified panel (*CALCOCO*2 + *SAMD9L* + *IFITM*3) more suitable for configuration in this current format was conducted. This showed good performance for discrimination of all TB disease groups from all controls (ROC = 0.9079, %sensitivity = 90.43, %specificity = 76.34), but individually only for the PTB (ROC = 95.22% sensitivity = 95.22, % specificity = 97.96, at a cut-off value of 7574 or ROC = 0.9894% sensitivity = 98.56, % specificity = 95.92, at a cut-off value of 10389) and J-EPTB groups (ROC = 0.933, % sensitivity = 90.43, % specificity = 78.13, at a cut-off value of 6201). It therefore demonstrated useful performance for discrimination of the PTB disease group at both the minimum and optimal WHO triage TPP requirements, for the J-EPTB group at the minimum WHO triage TPP requirement only and the minimum confirmatory test requirement for both. Cut-off thresholds could be adjusted to bias/maximise either the sensitivity or the specificity of the test for PTB diagnosis. This minimum panel shows potential, mainly as a triage test for PTB, but may additionally pick up high CALCOCO2, SAMD9L and IFITM3 expressing EPTB.

Other groups have conducted a recent systematic review of TB Biomarkers and multiple biomarker signatures: MacLean et al. evaluated the quality of biomarker studies and identified most promising biomarkers of active TB for development of

POC tests (71). Eleven host protein, blood-based studies were found to meet the WHO's TPP criteria. However, it was observed that most studies were of insufficient size or did not include a clinically relevant control population, resulting in over-inflated diagnostic performances. Competing blood-based biomarker signatures with potential for development often consisted of multiplex panels of more than 6 biomarkers, currently suboptimal for development of low cost POC triage tests. Jacobs et al. published a 6-host biomarker signature able to diagnose TB disease with a sensitivity of 100% and specificity of 89.3% (34). This 6-host biomarker signature included acute phase proteins CRP, ferritin and PCT typically associated with general inflammation. Although promising, the authors recognised the limitations of the size of the study. A larger cohort of patients was later published with a 7-host biomarker blood-based signature (72), however this study did not include extra-pulmonary TB patients. A similar study has recently been published by Garay-Baquero et al. (30), who identified a 5-panel plasma protein biomarker panel, which could distinguish TB from healthy controls (AUC = 0.93) and other respiratory diseases with a good degree of accuracy (AUC = 0.81).

In the study by Garay-Baquero et al. they state that *'Current limitations to mainstream serum or plasma proteomics pipelines partly stem from the predominance in protein mass (>95%) of the top 20 most abundant proteins. These high-abundance proteins either mask the presence of or are noncovalently bound to lower abundance proteins with potential clinical relevance. In an effort to overcome this limitation, an initial serum/plasma depletion step to remove such high-abundance proteins is typically employed before the mass spectrometry–based analysis. This plasma proteome analysis strategy has been used in samples from patients with TB. However, this approach will result in the inadvertent loss of a wide spectrum of physiologically important proteins, including those typically encountered in lipid microvesicles, such as exosomes, proteases and their cleavage products, and native peptides such as hormones.* These workers adopted a multidimensional or orthogonal liquid chromatographic separation combined with high-definition mass spectrometry analysis, to enable discovery of low–molecular weight sub-proteome protein targets.

This and many previously published protein target studies have used plasma or serum as sample type. This analytical approach is unlikely to detect peripheral blood, cell-bound targets, unless from lysed cells or if found free in soluble form. Many proteins are likely to be removed during the centrifugation procedure used to generate plasma and serum including cell-associated or cell-bound targets. In this study the sample type used was whole lysed blood. The results presented here may reflect an alternate cell-associated proteome, which is not easily assayed using other methods e.g. mass spectrometry. Cross-comparison of our results with other studies is likely therefore to be discordant, due to these differences in sample type and preparation and analytical methodology.

However, the targets identified in the Garay-Baquero study after validation using ELISA or Luminex assays are observed to exhibit expression levels in a similar concentration range to the protein targets presented in this study (high ρg to ηg/ml concentration range). There may be therefore synergies between different experimental approaches which could be optimised to facilitate future TB diagnostic assay development, particularly for development of a confirmatory test which meets all WHO optimum performance criteria for sputum positive TB (whose stringent criteria no panel has met so far). Further work is required to assess the reproducibility of the protein targets in this study prior to ongoing assay development, however the blood sampling and lysis technology is likely to fit well with POC or LFD-type technologies and assist with assay turnaround time and ease of use.

In summary, we have evaluated 9 biomarkers identified in our previous qPCR study for protein expression in whole lysed blood of TB patients and controls. Our 6-marker panel is showing promise for use for diagnosis of all forms of ATB in a laboratory setting e.g. on based multiplex immunoassays such as Luminex, Fireplex or ELISA or potentially future multianalyte LFDs. Utility of a simplified protein biomarker panel for use on POC devices e.g. current lower-complexity LFDs, showed limited potential for PTB and high expression EPTB patients only. No biomarker panel was identified which showed usefulness for discrimination of LTBI from demographically appropriately matched controls, although a significant difference in CD52 expression was observed in LTBI progressor. However this is unlikely to be useful in regions with a high background of endemic HIV. Further work is required to develop these panels further on suitable platforms and devices.

6 STUDY LIMITATIONS

A major limitation of this study was the logistics relating to ELISA assay procurement, shipping and experimental evaluation in two different laboratory settings, with different ambient environments and equipment, leading to some assay replicate failures. Future studies should also include samples from confirmed non-TB mycobacterial infectious or inflammatory diseases such as pneumonia due to other bacterial infectious agents e.g. group A Streptococci, sarcoidosis and other similar systemic inflammatory disorders as well as uninfected groups, to ascertain the specificity of the diagnostic panels for TB. The number of LTBI individuals progressing to active disease was also relatively small and this limited the power of the analysis, as they could not be analysed as a separate group with some of the statistical methods used. Limited demographic information was available for the control volunteers and patients included in the study including age and sex, for the patients HIV, Hepatitis B and C and CMV status and for the control groups incomplete verification of TB, HIV, Hepatitis B and C and CMV status. Future studies would be planned to address these issues and capture this information.

DATA AVAILABILITY STATEMENT

The datasets presented in this study can be found in online repositories. The names of the repository/repositories and accession number(s) can be found below: https://doi.org/10.6084/m9.figshare.19208328.v2.

ETHICS STATEMENT

The studies involving human participants were reviewed and approved by the JIPMER Institute Ethics committee (Human studies), AIIMS Institute Ethics committee and PHE, UK (India Study Number JIP/IEC/2015/11/522, UK Study Number PHE0186). The patients/participants provided their written informed consent to participate in this study.

AUTHOR CONTRIBUTIONS

HG, KE, MH, NW, PP SP, and KK conducted the experimental work. JS, SK, HB, IA, SS, and NJ provided control and patient samples and clinical and scientific expertise to the project. KE, NJ, SV, PP, and KK designed the study protocol and managed the study. HG, KE and KK conducted the data analysis. HG, KE, NJ, SV, and KK wrote and edited the paper. All authors contributed to the article and approved the submitted version.

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SUPPLEMENTARY MATERIAL

The Supplementary Material for this article can be found online at: https://www.frontiersin.org/articles/10.3389/fimmu.2022.854327/full#supplementary-material

Supplementary Table 1 | Table S1.1. Details of Patient and Control Samples **Table S1.2** Summary of normalised ELISA data for all samples included in the study **Table S1.3** Summary statistics for al ELISA data from the UK-CNTRL group **Table S1.4** Summary statistics for al ELISA data from the P-CNTRL group **Table S1.5** Summary statistics for al ELISA data from the A-CNTRL group **Table S1.6** Summary statistics for al ELISA data from the A-EPTB group **Table S1.7** Summary statistics for al ELISA data from the UK-CNTRL group **Table S1.8** Summary statistics for al ELISA data from the LTBI group **Table S1.9** Summary statistics for al ELISA data from the PTB group **Table S1.10** Summary of the mean, median and standard deviation biomarker expression values across all groups **Table S1.11** Summary of the biomarker expression paired t-test p-values across all groups Summary statistics for al ELISA data from the PTB group **Table S1.12** Summary of the average, fold-change and p-values for biomarker expression for the P-CNTRL, LTBI_PR and LTBI_NPR groups **Table S1.13** Correlation of biomarker target expression with QFT-GIT, T.SPOT and TST positivity in the combined LTBI group. **Table S1.14** Summary statistics for biomarker expression correlation matrices across all biomarkers and groups.

Supplementary Table 2 | Table S2.1 Random Forest modelling analysis for classification of Active TB and PTB from all combined control groups **Table S2.2** Single biomarker ROC curve analyses across all pairwise group combinations **Table S2.3** 5- and 3-biomarker panel ROC curve analyses across control and active TB disease groups. **Table S2.4** 5- and 3-biomarker panel ROC curve analyses across control and active TB disease groups and performance according to WHO triage test performance characteristics, plus positive and negative predictive values **S2.5** 5- and 3-biomarker panel ROC curve analyses across control and active TB disease groups and performance according to WHO confirmatory test performance characteristics, plus positive and negative predictive values.

Supplementary Information File S1 | Figure S1.1. Average and standard deviation for biomarker expression across active TB and control groups; Δ UK-CNTRL, ■ P-CNTRL, □ A-CNTRL, ◊ A-EPTB, ◆ J-EPTB, ● PTB **Figure S1.2**. Average and standard deviation biomarker expression across active TB and control groups; ○ UK-CNTRL, ● A-CNTRL, ▲P-CNTRL, ■ LTBI_NPR, □ LTBI_PR.

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Immunologic Biomarkers in Peripheral Blood of Persons With Tuberculosis and Advanced HIV

Artur T. L. Queiroz[1,2†], Mariana Araújo-Pereira[1,2,3,4†], Beatriz Barreto-Duarte[1,2,5,6†], Adriano Gomes-Silva[7,8†], Allyson G. Costa[9], Alice M. S. Andrade[1,2,3], João Pedro Miguez-Pinto[1,2,5], Renata Spener-Gomes[9], Alexandra B. Souza[9], Aline Benjamin[7], Flavia Sant'Anna[7], Marina C. Figueiredo[10], Vidya Mave[11,12], Padmini Salgame[13], Jerrold J. Ellner[13], Timothy R. Sterling[10], Marcelo Cordeiro-dos-Santos[9], Bruno B. Andrade[1,2,3,4,5,6,10,14‡] and Valeria C. Rolla[7‡]*

[1] Instituto Gonçalo Moniz, Fundação Oswaldo Cruz, Salvador, Brazil, [2] Multinational Organization Network Sponsoring Translational and Epidemiological Research Initiative, Salvador, Brazil, [3] Curso de Medicina, Universidade Faculdade de Tecnologia e Ciências (UNIFTC), Salvador, Brazil, [4] Faculdade de Medicina, Universidade Federal da Bahia, Salvador, Brazil, [5] Curso de Medicina, Universidade Salvador (UNIFACS), Salvador, Brazil, [6] Programa de Pós-Graduação em Clínica Médica, Universidade Federal do Rio de Janeiro, Rio de Janeiro, Brazil, [7] Instituto Nacional de Infectologia Evandro Chagas, Fundação Oswaldo Cruz, Rio de Janeiro, Brazil, [8] Laboratório Interdisciplinar de Pesquisas Médicas, Fundação Oswaldo Cruz, Rio de Janeiro, Brazil, [9] Instituto de Pesquisa Clínica Carlos Borborema, Fundação de Medicina Tropical Doutor Heitor Vieira Dourado, Manaus, Brazil, [10] Division of Infectious Diseases, Department of Medicine, Vanderbilt University School of Medicine, Nashville, TN, United States, [11] Byramjee-Jeejeebhoy Government Medical College-Johns Hopkins University Clinical Research Site (BJGMC-JHU CRS), Pune, India, [12] School of Medicine, Johns Hopkins University, Baltimore, MD, United States, [13] Rutgers- New Jersey Medical School, Center for Emerging Pathogens, Newark, NJ, United States, [14] Curso de Medicina, Escola Bahiana de Medicina e Saúde Pública (EBMSP), Salvador, Brazil

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***Correspondence:**
Bruno B. Andrade
Bruno.andrade@fiocruz.br

[†]These authors have contributed equally to this work and share first authorship

[‡]These authors have contributed equally to this work and share senior authorship

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Introduction: Tuberculosis (TB) is a common opportunistic infection among people living with HIV. Diagnostic tests such as culture, Xpert-MTB-RIF, and ULTRA have low sensitivity in paucibacillary TB disease; a blood biomarker could improve TB diagnostic capabilities. We assessed soluble factors to identify biomarkers associated with TB among persons with advanced HIV.

Methods: A case-control (1:1) study was conducted, with participants from Rio de Janeiro and Manaus, Brazil. People living with HIV presenting with CD4 count ≤100 cells/mm3 were eligible to participate. Cases had culture-confirmed TB (N=15) (positive for *Mycobacterium tuberculosis* [Mtb]); controls had HIV-infection only (N=15). Study visits included baseline, month 2 and end of TB therapy, during which samples of peripheral blood were obtained. A panel containing 29 biomarkers including cytokines, chemokines and growth factors was utilized to assess candidate biomarkers using Luminex technology in cryopreserved EDTA plasma samples. We used neural network analysis, based on machine learning, to identify biomarkers (single or in combination) that best distinguished cases from controls. Additional multi-dimensional analyses provided detailed profiling of the systemic inflammatory environment in cases and controls.

Results: Median CD4 count and HIV-1 RNA load values were similar between groups at all timepoints. Persons with TB had lower body mass index (BMI) (median=19.6, Interquartile Range [IQR]=18.6-22.3) than controls (23.7; IQR: 21.8 = 25.5, p=0.004). TB coinfection was also associated with increased frequency of other comorbidities. The overall profile of plasma cytokines, chemokines and growth factors were distinct between

the study groups at all timepoints. Plasma concentrations of IL-15 and IL-10 were on average lower in TB cases than in controls. When used in combination, such markers were able to discriminate between TB cases and controls with the highest degree of accuracy at each study timepoint.

Conclusion: Among persons with advanced HIV, plasma concentrations of IL-15 and IL-10 can be used in combination to identify TB disease regardless of time on anti-TB treatment.

Keywords: biomarkers, diagnosis, tuberculosis, advanced HIV, IL-15, IL-10, cytokine

INTRODUCTION

By the end of 2019, it was estimated that there were 38 million people living with HIV (PLWH) in the world, In 2020, there were still 690,000 HIV-related deaths and 1.7 million new infections globally (1). Without treatment, PLWH typically progress through several stages. When CD4 cell counts are <200 cells/mm^3 or patients are classified as WHO stage 3 or 4, the disease is characterized as advanced, with a very high risk of death (1).

It is common for people with advanced HIV (PLWH) to experience opportunistic infections. One of the most common co-infections is tuberculosis (TB). Approximately 10 million people have had TB in 2019 globally, of whom 8% were co-infected with HIV (2). Co-infection with HIV and TB accelerates the progression of both diseases and leads to substantial deterioration of effective immune responses, significantly impacting morbidity and mortality (3–6). TB-HIV coinfection was responsible for approximately 214,000 deaths in 2020 (2).

TB diagnosis has improved recently due to the availability of rapid molecular assays such as Xpert MTB/RIF (76.5% sensitivity) and Xpert MTB/RIF ULTRA (up to 88.2% sensitivity). However, the accuracy of such diagnostic tests is still limited, particularly in PLWH, who often have paucibacillary disease and therefore negative screening for acid-fast bacilli (AFB) in sputum smears (7). In such patients, the sensitivity of ULTRA is reduced to 64.7%, and the sensitivity of Xpert MTB/RIF is 41.2% (8). On the other hand, HIV increases by at least 20-fold the risk of progression from latent *M. tuberculosis* (Mtb) infection to active TB (9). As a result, PLWH with advanced disease exhibit a high risk of incident active TB (10). Understanding how the immune response modulates Mtb infection and progression to TB disease is crucial to develop immunologic tools to identify TB in PLWH (10). Given that many immunologic proteins are regulated during Mtb infection, in the present study we tested whether quantification of such plasma molecules could serve as informative TB biomarkers in PLWH.

METHODS

Ethical Statement

This study was approved by the Institutional Review Boards from each clinical site (CAAE: 85790218.4.1001). All participants signed an informed consent form before enrollment. All clinical and laboratory data were de-identified to protect participants' confidentiality. All clinical investigations were conducted according to the principles of the Declaration of Helsinki.

Study Design and Eligibility Criteria

This was a case-control study (1:1) to evaluate the performance of blood soluble molecule signatures associated with TB among PLWH with advanced disease. Cases and controls (15 participants in each group) were recruited from February 2018 to December 2019, based on study eligibility criteria. Participants were recruited if they agreed to participate, provided informed consent, were ≥18 years old, HIV-positive, and had CD4 count ≤100 cells/mm^3. TB had to be confirmed by mycobacteriologic culture for the TB-HIV group. In the controls with HIV only, participants had no signs or symptoms of TB and were screened negative with smear and culture examination. Participants were excluded if they were pregnant or had any condition that, in the judgment of the investigator, precluded participation because it could have affected the subject's safety. To be eligible for the TB-HIV group, TB treatment could have been initiated no more than 7 days before enrolment. Study participants were recruited from one site in Rio de Janeiro (INI- Fiocruz) and one site in Manaus (FMT), both in Brazil. These sites have active TB cohorts and affiliated HIV treatment centers.

Clinical and sociodemographic data and plasma samples were obtained at baseline, month 2 and end of therapy or equivalent timepoint in the control group (END visit). CD4 count and HIV-1 RNA (viral load; VL) were performed at all study visits (baseline, Month 2 and END of therapy). All participants (n=15, 100%) in the HIV group and 6 (40%) in the TB-HIV group were receiving antiretroviral therapy (ART) at enrollment; 9 (60%) TB-HIV patients initiated ART during TB therapy. TB-HIV patients were classified as having either pulmonary (PTB) or pulmonary + extrapulmonary (PTB+EPTB). None of the participants had only EPTB.

Measurements of Plasma Biomarkers

All plasma samples, stored at -80°C, were quantified for 29 biomarkers (cytokines, chemokines and growth factors) using a commercially available Luminex kit (Millipore): Epidermal growth factor (EGF), EOTAXIN, Granulocyte colony-stimulating factor (G-CSF), Granulocyte-macrophage colony-stimulating factor (GM-CSF), Interferon (IFN)-α2, IFN-

gamma, Interleukins (IL)-10, IL-12P40, IL-12P70, IL-13, IL-15, IL-17A, IL-1RA, IL-1a, IL-1B, IL-2, IL-3, IL-4, IL-5, IL-6, IL-7, IL-8, CXCL10, Monocyte Chemoattractant Protein (MCP)-1/CCL2, Macrophage Inflammatory Protein(MIP)-1a/CCL3, MIP-1b/CCL4, Tumor Necrosis Factor (TNF)-α, TNF-β and Vascular Endothelial Growth Factor (VEGF).

Heatmaps

A one-sided unsupervised hierarchical clustering [Ward's method (11)] and a heatmap representation were used to display z-score normalized log_2-transformed data and depict the overall profile of samples and biomarkers. In this analysis, the dendrograms represent the Euclidean distance (inferring degree of similarity). This analysis was performed using the *ComplexHeatmap* R package (12). Moreover, the log_2 fold change was measured, and the significance was calculated with the *t.test* (13) and corrected with the False Discovery Ratio (FDR) (14).

Machine Learning Modeling

After the data preparation, we applied an averaged Neural Network (ANN) model (15), with repeated 20-fold cross-validation to classify the outcomes TB-HIV and HIV with the *caret* R package (16). The best variables were retrieved and used on a linear model for evaluation. The most informative variables were retrieved from the dataset and the classification was assessed by receiver operating characteristic (ROC) curves and the area under the curve (AUC) value ((17)).

Network Analysis

Correlation analyses were performed using the Spearman's rank test (18), using the rcorr function from the *Hmisc* R package (available at: https://rdrr.io/cran/Hmisc/). Only significant correlations ($p<0.05$) were depicted in the network. The correlation networks were constructed using the *igraph* R package (19). All analyses were performed in R (version 4.0.2). To evaluate the network reliability, we also applied bootstraps 20 with 100 replicates (20). We retrieved each network density and the degrees of each edge. The differences between the groups were tested with a t-test.

RESULTS

There were 15 TB-HIV cases and 15 controls with HIV. All 15 TB cases had pulmonary disease; 8 also had extrapulmonary disease: lymph node (n=1)intestinal (n=2), splenic (n=1), laryngeal (n=1), cutaneous (n=1), renal (n=1), and genitourinary (n=1). No cases of immune reconstitution inflammatory syndrome (IRIS) were diagnosed during the study.

Cases and controls had similar clinical and demographic characteristics, though there were some differences (**Table 1**). Body mass index (BMI) was lower in TB-HIV cases (median=19.6, Interquartile Range [IQR]=18.6-22.3) than HIV controls (23.7; IQR: 21.8 = 25.5, p=0.004). However, $CD4^+$ count and HIV viral load (VL) were similar between the groups at baseline and during follow-up (**Figures 1A, B**).

TABLE 1 | Characteristics of people living with advanced HIV by TB status.

Characteristics	TB-HIV (n=15)	HIV (n=15)	p-value
Age – median (IQR)	37.0 (30.5-38.5)	33.7 (30.2-43.2)	0.884
Male sex – no. (%)	10 (66.7)	12 (80.0)	0.682
Race/Ethnicity – no. (%)			0.337
White	2 (13.3)	5 (33.3)	
Black	3 (20.0)	2 (13.3)	
Pardo	8 (53.3)	8 (53.3)	
Indigenous	2 (13.3)	0 (0.0)	
BMI – median (IQR)	19.6 (18.6-22.3)	23.7 (21.8-25.5)	**0.004**
CD4 count – median (IQR)	53.0 (38.5-67.0)	63.0 (44.5-71.0)	0.290
Log10 HIV Viral Load – median (IQR)	5.33 (4.53-5.61)	5.23 (3.98-5.73)	0.113
ART – no. (%)	6 (40.0)	15 (100)	**0.001**
Smoking – no. (%)	6 (40.0)	3 (20.0)	0.427
Passive smoking – no. (%)	4 (26.7)	0 (0)	0.100
Alcohol consumption – no. (%)	13 (86.7)	12 (80.0)	1.000
Illicit drug use – no. (%)	4 (26.7)	2 (13.3)	0.651
Prior TB episode– no. (%)	3 (20.0)	0 (0.0)	0.224
Cancer– no. (%)	0 (0.0)	0 (0.0)	NA
Diabetes– no. (%)	3 (20.0)	0 (0.0)	0.224
Renal disease– no. (%)	0 (0.0)	0 (0.0)	NA
Hypertension– no. (%)	0 (0.0)	0 (0.0)	NA
Any comorbidity– no. (%)	15 (100)	8 (53.3)	**0.006**

Data represent no. (%), except for age and BMI, which is presented as median and interquartile range (IQR). Continuous variables were compared using the Mann-Whitney U test and categorical variables were compared using Fisher's exact test (2x2) or Pearson's chi-square test. Statistically significant differences (p-value < 0.05) are highlighted using bold-type font. Definition of alcohol consumption: Past or current any consumption of alcohol. Definition of passive smoking: Living with someone who smokes. Definition of illicit drug use: Past or current illicit drug use (marijuana, cocaine, heroin or crack). Definition of Pardo ethnicity: mixture of European, black and Amerindian.

TB, tuberculosis; BMI, Body Mass Index; NA, Not applicable; ART, antiretroviral therapy.

Any comorbidity: mycosis, syphilis, retinitis, allergies.

Of note, CD4 counts increased over time in both groups (HIV: p=0.009; TB-HIV: p<0.001) (**Figure 1C**). However, the fold change in $CD4^+$ count within each study group over time was different. The $CD4^+$ count increase was statistically significant between baseline and month 2 in the TB-HIV group (p<0.001); and baseline *vs* END visit (p<0.001) in both groups (**Figure 1C**). The decrease in VL over time was statistically significant only in the TB-HIV group (p=0.003). The fold-change was significant when comparing baseline *vs* month 2 (p<0.001) in the TB-HIV group; and baseline *vs* END visit (p<0.001) in both groups (**Figure 1D**). Taken together, the results suggest that in PLWH, those with TB exhibited a laboratory profile indicative of more advanced disease than PLWH without TB. However, as treatment progressed, this profile became similar to that observed in the HIV control group.

Next, we performed multi-dimensional analyses to assess the systemic inflammatory profile of each group. First, we used an unsupervised hierarchical cluster analysis to identify inflammation profiles of distinct groups at each timepoint. It was possible to identify two main clusters at all timepoints; in general, TB-HIV patients had a distinct profile at all timepoints, with lower levels of biomarkers than HIV controls **(Figure 2).** The levels of all biomarkers measured are summarized in **Supplementary Table 1**. At baseline, this difference was significant for IL-15 (Fold Change [FC] TB-HIV vs. HIV: -3.592, p<0.001), IL-17A (FC: -2.19, p=0.044) and IL-2 (FC: -2.20, p=0.023) (**Figure 2A**). At month 2 of anti-TB therapy, this difference was significant in IL-15 (FC: -4.51, p=0.004), IL-2 (FC: -3.44, p=0.048) and IL-3 (FC: -3.98, p=0.032) (**Figure 2B**). Furthermore, at END visit, TB-HIV participants exhibited on average diminished levels of Eotaxin (FC: -1.55, p=0.0038), GM-CSF (FC: -2.89, p=0.048), IL-15 (FC: -3.45, p<0.001) and TNF-α (FC: -2.14, p<0.007) (**Figure 2C**). We also showed the fold-change values for baseline, month 2 and END visit timepoints in the respective **Supplementary Table 3; Tables 4, 5**. IL-2 showed a significant difference between time points: baseline and month 2 while IL-15 was the only cytokine that was significantly lower at both timepoints (baseline, month 2 and END visit) (**Figure 2**). Additionally, we performed comparisons of biomarker levels in TB-HIV patients according type of TB (PTB: n=7; PTB+EPTB: n=8) and observed that there were no differences in biomarker levels between these two groups, except for EGF, which was higher in PTB (p=0.048) than in PTB+EPTB (**Figure 3**). The values of markers in each group are described in **Supplementary Table 2**. We also compared HIV patients according to comorbidities (i.e., mycosis, syphilis, retinitis, allergies, cancer, diabetes, renal disease, hypertension), and observed that those with any comorbidity presented higher values of two cytokines in comparison with those without comorbidities: IFN-gamma (Any comorbidity: 3.43, IQR:2.80-4.27; Without Comorbidity: 2.0, IQR: -2.09-2.33; p=0.024) and IL-4 (Any comorbidity: 3.23, IQR: -1.95-43.78; Without Comorbidity: -6.64, IQR:-6.64—6.63; p=0.015) (**Figure 4**).

Next, we used neural network analysis, an approach based on machine learning (16), to identify the marker or combination of markers that could be more informative to distinguish cases from controls. Using this approach, we identified IL-15 and IL-10 as the best classifiers to distinguish groups at all the study timepoints (**Figure 5**). The thresholds were 5.13 pg/ml for IL-15 and 1.14 pg/ml for IL-10. Indeed, the distribution of these biomarkers could fairly classify the samples from each group at baseline, month 2 and END visits (**Figure 5A**). We also verified the individual variation of IL-15 and IL-10 at the different timepoints and found that although the concentrations of both biomarkers were substantially diverse in the study population, TB-HIV participants in general exhibited lower concentrations throughout the entire study period (**Figure 5B**). The best classifiers identified in the neural network analysis were retrieved and we tested their classification performance at the different timepoints using ROC curves. Using this approach, we

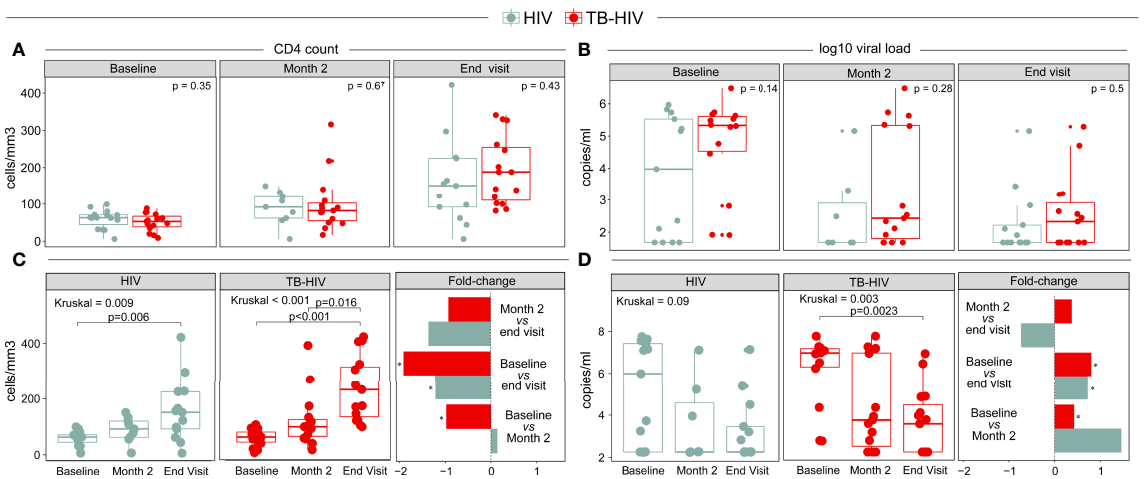

FIGURE 1 | Differences in **(A, C)** $CD4^+$count (cells/mm^3) and **(B, D)** HIV viral load (HIV RNA copies/mL) between the TB-HIV and HIV groups at different timepoints. *Represents p-values < 0.001.

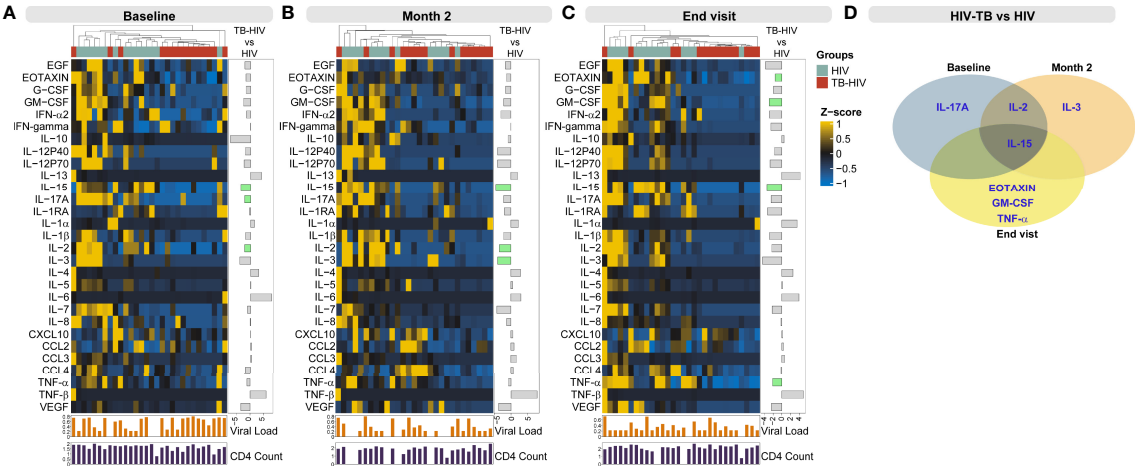

FIGURE 2 | Profiling systemic inflammation to identify markers differentially expressed in persons with TB-HIV coinfection. Concentrations of the indicated biomarkers were quantified in plasma samples using Luminex technology in study participants at baseline (prior to anti-TB treatment) **(A)**, at month 2 **(B)** and at END visit **(C)** of anti-TB treatment. Data were log2-transformed, and z-score normalized to build the heatmaps at each study timepoint. A hierarchical cluster analysis (Ward's method, with dendrograms implying the Euclidean distance) was employed to test whether combined quantification of plasma concentrations of the biomarkers could be used to segregate the TB-HIV from the HIV group. The upper colored line corresponds to the groups: in light blue are the HIV samples and in red are the TB-HIV samples. The below bar plots correspond to log10 Viral load and $CD4^+$ count. The side bar plot shows the fold change values from the comparison TB-HIV vs HIV and green bars are the differences with significant false discovery rate (5%). **(D)** Venn diagram with intersection with markers that showed statistical significance when comparing TB-HIV with HIV. All significant comparisons showed a negative fold-change.

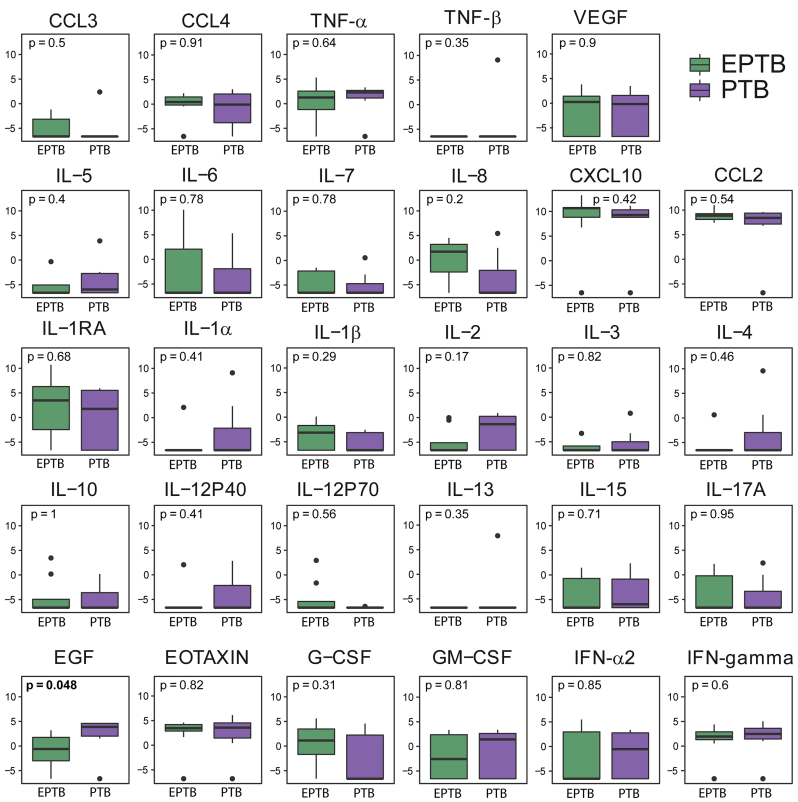

FIGURE 3 | Soluble inflammatory markers do not differ according to the location of TB in TB-HIV patients. Boxplots show the levels of each marker analyzed at baseline, comparing pulmonary TB (PTB, n = 7) with PTB+extrapulmonary TB (EPTB, n = 8) in the TB-HIV population (n = 15). Only EGF showed a statistically significant difference ($p<0.05$) in comparisons.

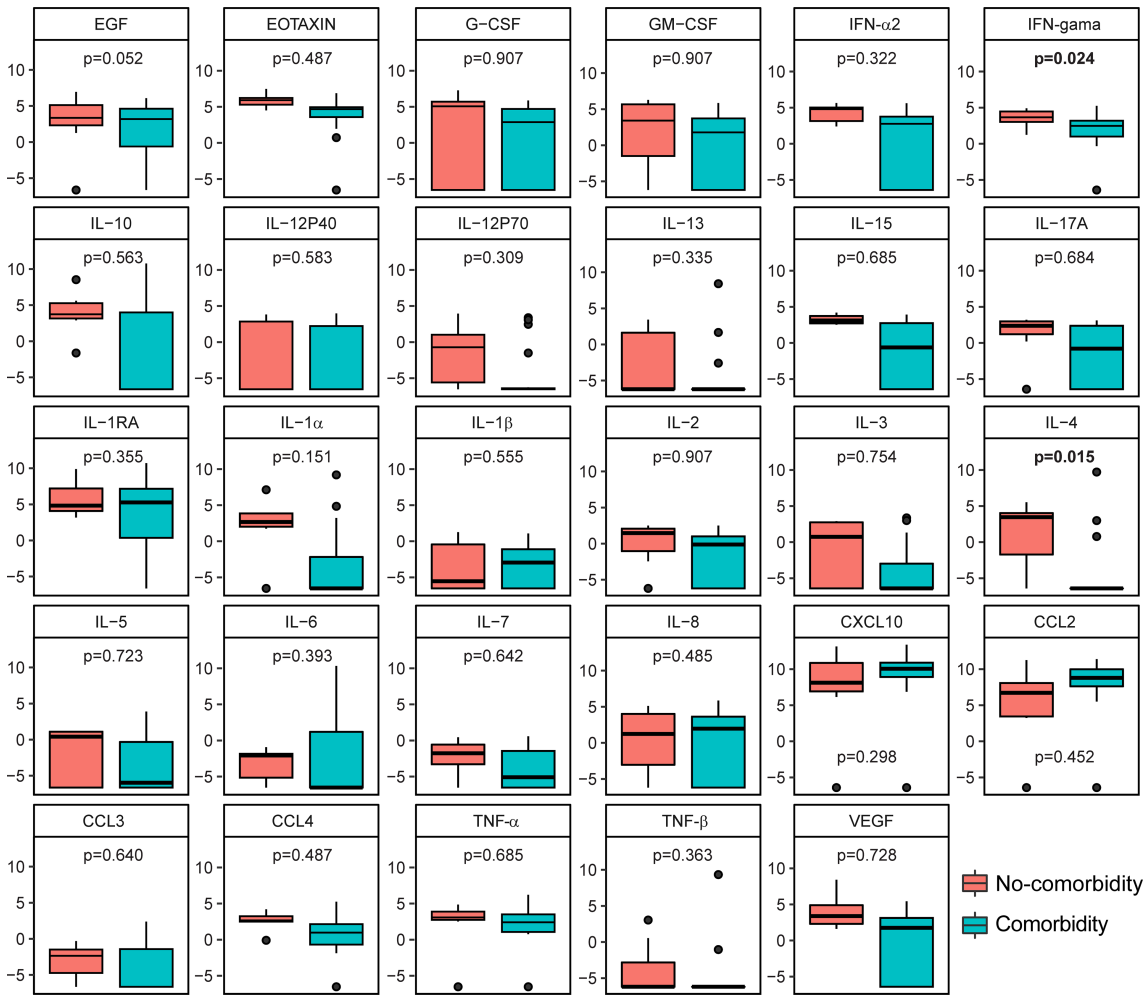

FIGURE 4 | Soluble inflammatory markers differ according to comorbidity occurrence in HIV patients. Boxplots show the levels of each marker analyzed at baseline, comparing comorbidity occurrence (n = 8) with those without comorbidity (n = 7) in the HIV population (n = 15). Only IFN-gamma and IL-4 showed a statistically significant difference ($p < 0.05$) in comparisons.

identified that the cytokines showed better performance at baseline (area under the curve [AUC]: 1.0, p<0.0001), followed by a slight decrease in performance at month 2 (AUC: 0.964, p<0.0001) and a further decrease at END visit (AUC: 0.950, p<0.0001) (**Figure 5C**).

We observed that time on ART was significantly different between the groups (p=0.005), The HIV control group had a longer time on ART (4.14; IQR:1.14-21 weeks) compared to TB-HIV cases (-0.14, IQR: 1.92-6.36 weeks) (**Figure 6A**). At study initiation, the entire HIV control group was already on ART, and only 6 of the TB-HIV group were on ART. Nine of 15 patients in the TB-HIV group started ART after enrollment (**Figure 6B**). To ensure that time on ART was not influencing the levels of these biomarkers, we performed linear regressions with both measures (time on ART versus IL-15 and time on ART versus IL-10), which demonstrated that there were no significant correlations between time on ART and the levels of such cytokines in peripheral blood (**Figures 6C, D**).

To understand the coordination of immune responses in the peripheral blood (21) we used network analysis to assess correlations between the concentrations of the biomarkers, CD4 counts, VL, and BMI. This analysis revealed that the network profiles of the different clinical groups at each study timepoint were very distinct (**Figure 7**). At month 2, VL was positively correlated with IFN-gamma , IL-12p70, IL-15 and IL-5 (**Figure 7**). At the END visit, CD4 counts were negatively correlated with IL-5. VL was positively correlated with EGF, IL-12p70, IL-17a and CCL3. BMI was negatively correlated with IL−2 (**Figure 7**). In the HIV-control group, VL was negatively correlated with BMI and G-CSF count, at baseline (**Figure 7**). At month 2, VL was positively correlated with G−CSF. BMI was negatively correlated with IL−15, CXCL10 and CCL2 (**Figure 7**). At the END visit, VL was negatively correlated with IL-1B, IL-2, IL-5, CCL3, G−CSF and BMI. CD4 count was negatively correlated with BMI and positively correlated with EGF and VEGF (**Figure 7**).

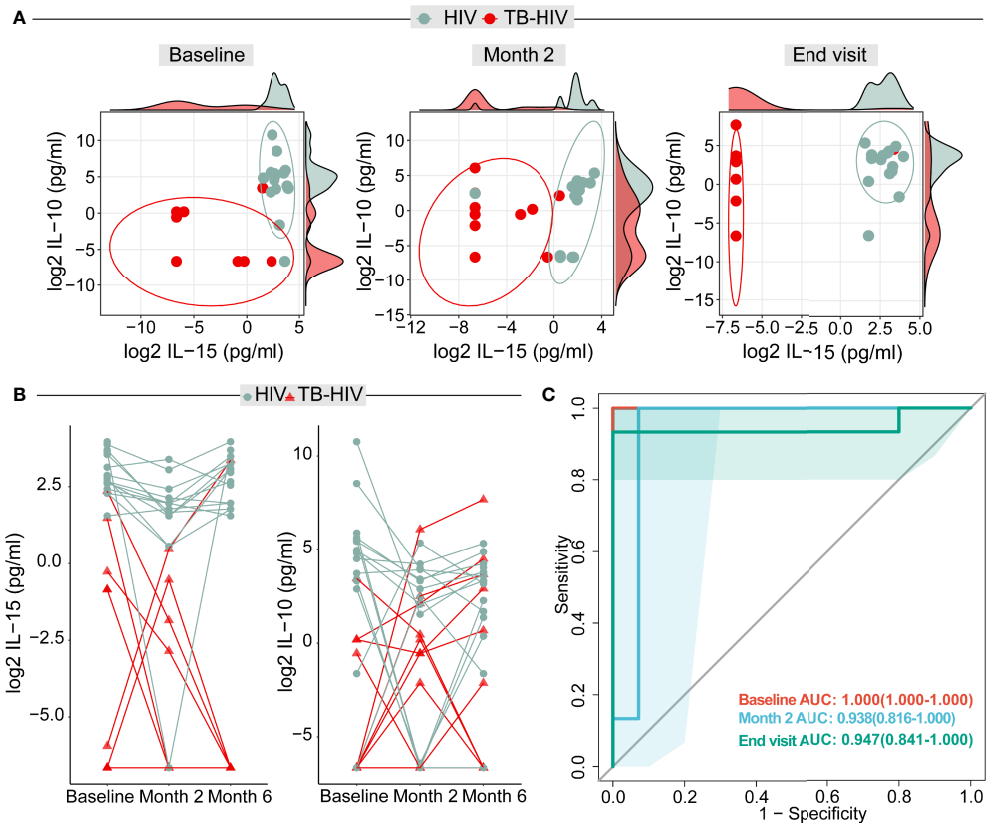

FIGURE 5 | Artificial neural network analysis identified IL-15 and IL-10 as potential markers for distinguishing TB cases among persons with advanced HIV. **(A)** Dot plot from the biomarker values in both TB-HIV and HIV groups in the baseline, month 2 and END visit. The ellipses were calculated with the "distance t" from each group. The axis parallels graphs are each IL-15 and IL-10 log2 values displayed in density distribution (histograms). **(B)** Distribution of biomarkers in plasma from TB-HIV and HIV participants: Dots represent individuals with HIV-infection, whereas the triangles indicate TB-HIV participants. Values from a given study participant at the indicated study timepoints are connected through colored lines. The y axis shows the log2 IL-15 and IL-10 values. **(C)** Receiver Operator Characteristics (ROC) analysis using the plasma concentrations of both IL-15 and IL-10 at each study timepoint was performed to test the accuracy of the combined markers in distinguishing TB-HIV from HIV cases. All the areas under the curves (AUC) exhibited p-value of <0.0001.

The HIV control group had significantly higher network density than TB-HIV cases at baseline (p<0.001) and at the END visit (p<0.001) (**Figure 8A**). Moreover, the IL-10 number of connections was higher in the HIV group compared with TB-HIV at the baseline (p<0.001) and END visits (p<0.001) (**Figure 8B**). Nevertheless, the IL-15 degree was significantly increased in TB-HIV at baseline (p<0.001) and END visits (p<0.001), suggesting that this molecule played a crucial role in the shift of network density between the groups, and regulating the immune response on the TB-HIV group (**Figure 8B**).

DISCUSSION

This is a study conducted in PLWH and advanced immunosuppression in both TB-HIV and HIV-infected participants. TB is an opportunistic infection (OI) that contributes to HIV replication and harm, being one of the most prevalent OI in the world and responsible for most deaths in HIV-infected population (22). As it is difficult to diagnose TB in advanced HIV due to the low sensitivity of diagnostic tests and X-rays. Moreover, signs and symptoms of TB are common to other OI and delays to diagnosis and treatment of TB can lead to hospitalization, death and other OI diagnosis. In this study we wanted to identify a biomarker that could segregate advanced HIV with TB from those without TB, using baseline characteristics, VL, CD4 count, and plasma soluble factors at baseline and follow up visits.

Our study found a lower BMI in the TB-HIV group showing the impact of weight loss to cellular immune response (23) that increase the risk to develop TB (24). Other comorbidities were more frequently seen in the TB-HIV group as well (p=0.006) which could increase morbidity and mortality. During follow-up CD4 counts were similar in both groups as well as VL although the reduction of VL was evident when comparing baseline values *vs* month 2 (p<0.001) in TB-HIV group; and baseline *vs* end visit (p<0.001) in both groups. Our results suggest that in PLWH, TB diagnosis is associated with a laboratory profile indicative of

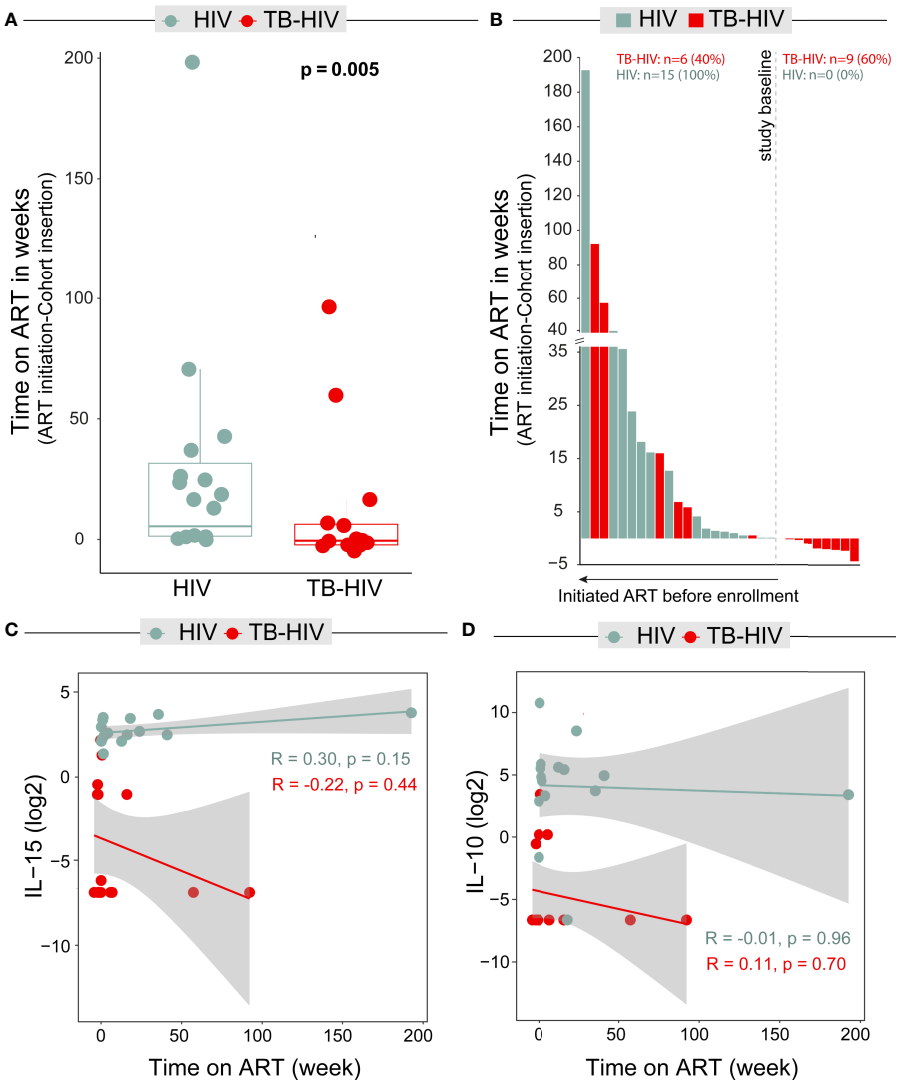

FIGURE 6 | Timing of antiretroviral therapy (ART) does not impact IL-15 and IL-10 levels. **(A)** Boxplot shows that those with TB-HIV had a shorter time between initiation of ARV and enrollment in the study in relation to HIV patients (Mann-Whitney test p = 0.005). **(B)** Bar graph shows that 8/15 (53.3%) TB-HIV started ARV before being enrolled. Figures **(C, D)** show linear regressions between ART time and IL-15 and IL-10 levels, respectively. A regression was performed for each group (TB-HIV- red and HIV - blue) in relation to each of the biomarkers. No statistically significant differences were found in any of the markers or groups.

more advanced disease than PLWH without TB. However, the TB-HIV group seems to gradually reach a similar profile observed in HIV-infected group.

Alternative assays for TB diagnosis should be recommended in cases of low sensitivity to detect Mtb by bacteriologic or molecular assays. In the case of PLWH approaches based on detection of blood proteins to diagnose TB have been already evaluated (10, 25). The search for an immunologic biomarker detected directly in plasma, and not after the (often overnight) stimulation assays, has the advantage to become a potential point-of-care rapid diagnostic test (26).

In the present work the two-marker host biosignature based on IL-15 and IL-10 plasma levels were able to define at baseline TB among PLWH with CD4 ≤100 cells/mm^3. Another work by Verma and colleagues (2018) done in Uganda founded plasma IFN-gamma as a good TB classifier (AUC 0.98) among PLWH (CD4 ≤100 cells/mm^3) (10). It was interesting to note that IFN-gamma was not among plasma cytokines profile able to classify TB in the present Brazilian PLWH groups. The genetic background of different populations possibly directly interferes with the ability to produce cytokines and reinforces the requirement for validation of potential biomarkers in different ethnicities. Another seven-marker (ApoA-1, CFH, CRP, IFN-gamma, CXCL10, SAA, and transthyretin) serum protein biosignature for the diagnosis of TB, irrespective of HIV-infection status or ethnicity in Africa, were identified as a promising biomarker for field friendly point-of-care screening test for pulmonary TB (27). However, tests based on large

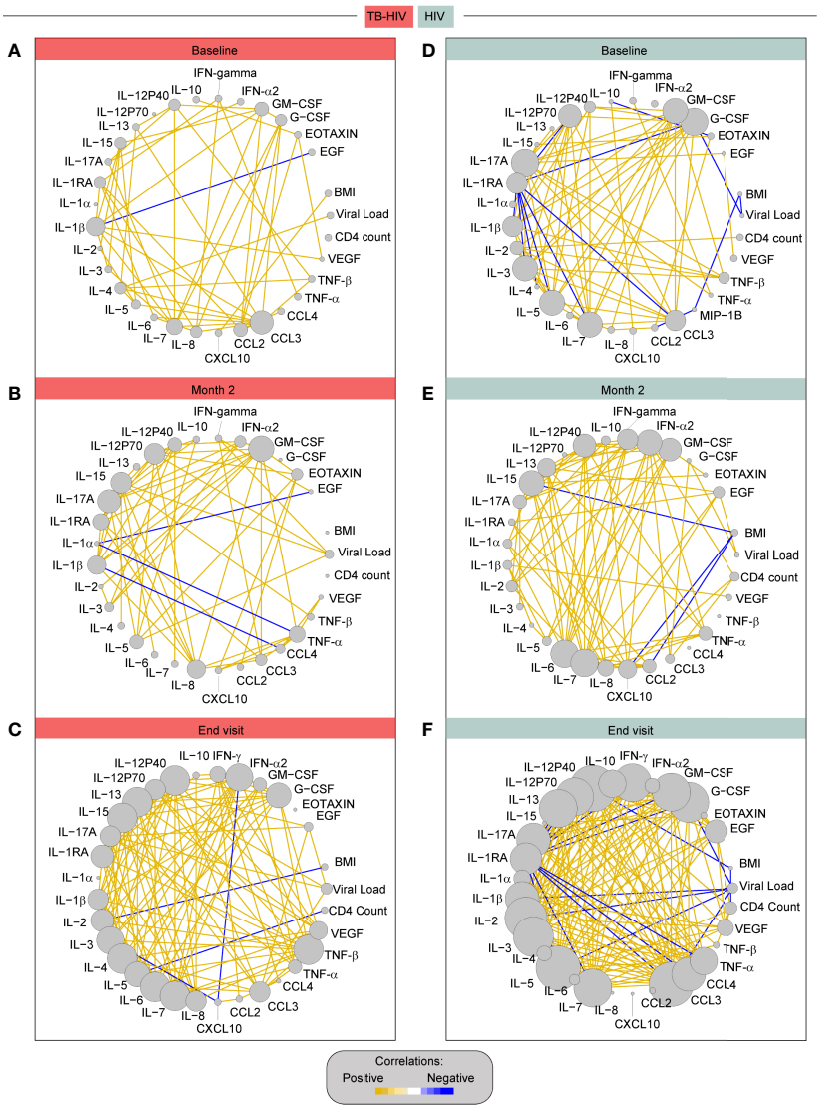

FIGURE 7 | Correlation network of TB-HIV and HIV groups across the timepoints. The diameter of each circle is proportional to the number of significant correlations prospectively. The connecting lines represent statistically significant correlations ($P < 0.05$). Yellow connecting lines represent positive correlations while blue lines infer negative correlations. **(A)** – TB-HIV group at baseline, **(B)** – TB-HIV group at month 2, **(C)** – TB-HIV group at END, **(D)** –HIV group at baseline, **(E)** – HIV group at month 2, and **(F)** – HIV group at end visit.

biosignatures are expensive and complex to be manufactured, making it difficult to use them as a point of care in the future (28). In a single protein approach, Yoon and colleagues (2017) have proposed for PLWH with CD4 <340 cells/mm^3 the quantification of C-reactive protein (CRP) by finger-prick blood sample, presenting 94% of sensitivity and 72% of specificity (25). Also, Lesosky and colleagues (2019) discussed the possibility to use plasma biomarkers based on immune response (IL-2 and IFN-gamma) as a predictive tool for TB progression among PLWH (29).

Although the present signature presents 100% of sensitivity and 100% of specificity it is not known whether non Mtb infection or other OI in association with HIV-infection could stimulate a similar cytokine profile and interfere with the specificity of this biomarker based on immunologic molecules. Also, this ability to classify TB into PLWH was maintained over time of TB treatment (month 2 and END visit) and is necessary to assess how long this signature will be maintained post treatment, since a false positive result could configure a limitation of this biomarker to identify active TB in PLWH with a previous history of TB treatment. As described before, diagnosis of TB patients with a previous history of TB are considered a challenge when using biosignatures based on the dosage of soluble blood factors (28).

The immune system coordination accessed by a network analysis of spearman correlations is an interesting approach to

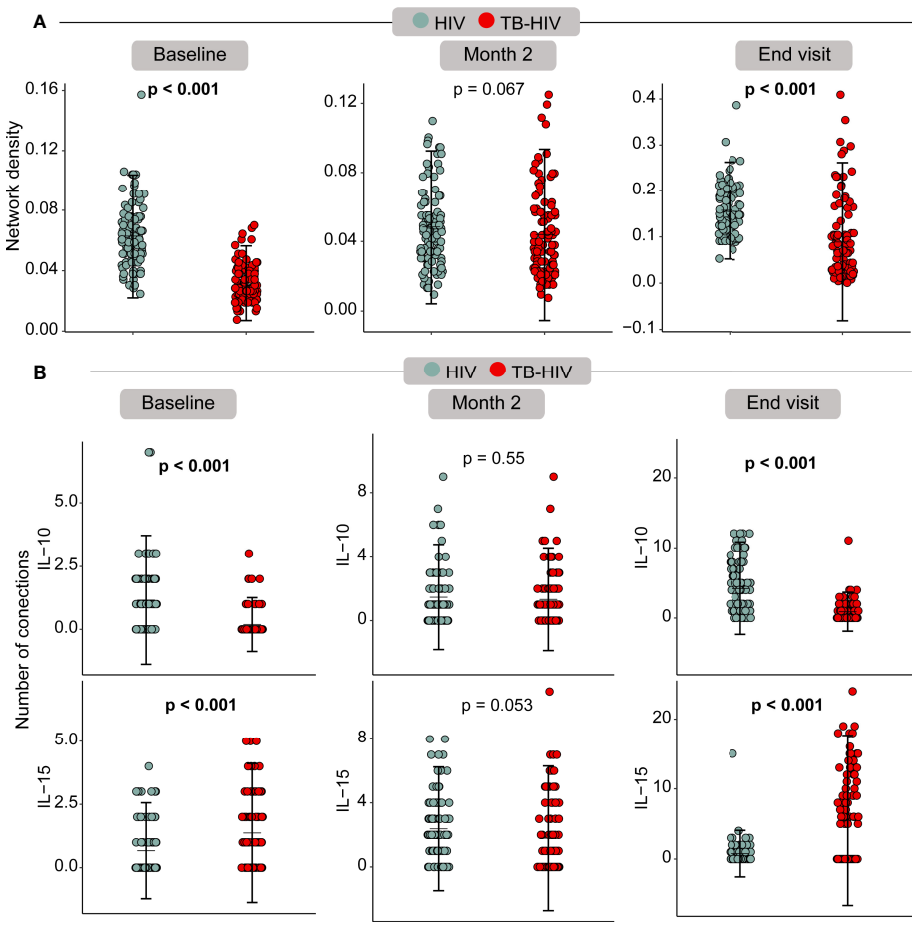

FIGURE 8 | Bootstrap correlation network analysis showed differences between baseline and END visits. **(A)** – Network density across the timepoints; **(B)** – Edge degree from IL-15 and IL-10 across the baseline, month 2 and end visit.

help understand biomarkers (30) that could be useful to classify TB among PLWH with advanced immunosuppression. At baseline it was clearly observed the lower network density by TB-HIV group in comparison to the HIV group. Coinfection with TB in PLWH probably imprints on the immune system a dysfunction of innate and adaptive immune cells that result in a significant dysregulation in cytokine production. At month 2 after TB treatment these differences between cases and controls disappear, which could be attributed by the Mtb control and the beginning recovery of the immune system homeostasis. At the END visit, the density network returned to be statistically higher in the HIV group in comparison to TB-HIV group, but specially for IL-15, the higher number of correlation interactions in TB-HIV group is suggestive that this cytokine has an important role in restoring the balance of the immune system after Mtb control.

Our study has limitations. This was an exploratory study with a small sample size with patients with only CD4 ≤100 cells/mm^3. Thus, additional comparisons exploring the potential interference of OIs in the performance of the discrimination model presented here were hampered due to underpowered statistical analysis. Thus, additional studies are warranted to define the relationships between OI and diagnosis performance of the proposed biosignature. Regardless, this study presents an pioneering approach that targets clinical and plasma immunological biomarkers for PLWH with advanced disease, and its results bring new insights that could be validated in other populations especially in other regions with distinct genetic and/or epidemiologic settings.

In conclusion we found in the present work that the combination of IL-15 and IL-10 plasma levels was able to define at baseline a biomarker signature associated with TB in PLWH and advanced immunosuppression. Although this test presents 100% of sensitivity and 100% of specificity this is an exploratory study with preliminary finding. Our results should be validated in bigger cohorts and other races.

DATA AVAILABILITY STATEMENT

The raw data supporting the conclusions of this article will be made available by the authors, without undue reservation.

ETHICS STATEMENT

The studies involving human participants were reviewed and approved by Institutional Review Board of the Instituto Nacional de Infectologia, Fundação Oswaldo Cruz (CAAE: 85790218.4.1001). The patients/participants provided their written informed consent to participate in this study.

AUTHOR CONTRIBUTIONS

AQ performed the analyses and wrote the first draft; MA-P Performed the analyses with AQ, integrated clinical and immunologic data and wrote the first draft; BB-D and AA performed the luminex experiment, curated the luminex output data, helped in data analysis and design of figures and wrote the first draft; AG-S and AC, data interpretation and wrote the manuscript discussion; RS-G and AS clinical team from Manaus; AB and FS clinical team from Rio de Janeiro; VM, PS, and JE India team; TS, MC, BA, and VR established the initial concept and wrote the manuscript. JE, PS, and VM established the initial concept and revised the manuscript. All authors contributed to the article and approved the submitted version.

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SUPPLEMENTARY MATERIAL

The Supplementary Material for this article can be found online at: https://www.frontiersin.org/articles/10.3389/fimmu.2022.890003/full#supplementary-material

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REVIEW
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Tuberculosis Phenotypic and Genotypic Drug Susceptibility Testing and Immunodiagnostics: A Review

Kizil A. Yusoof[1†], Juan Ignacio García[2†], Alyssa Schami[1,2], Andreu Garcia-Vilanova[2], Holden V. Kelley[2], Shu-Hua Wang[3], Adrian Rendon[4], Blanca I. Restrepo[5,6*], Marcel Yotebieng[7*] and Jordi B. Torrelles[1,2*]*

[1] Graduate School of Biomedical Sciences, University of Texas Health San Antonio, San Antonio, TX, United States, [2] Population Health Program, Tuberculosis Group, Texas Biomedical Research Institute, San Antonio, TX, United States, [3] Department of Internal Medicine, Division of Infectious Diseases, College of Medicine and Global One Health Initiative, The Ohio State University, Columbus, OH, United States, [4] Centro de Investigación, Prevención y Tratamiento de Infecciones Respiratorias (CIPTIR), Hospital Universitario de Monterrey Universidad Autónoma de Nuevo León (UANL), Monterrey, Mexico, [5] School of Public Health, University of Texas Health Science Center at Houston, Brownsville, TX, United States, [6] School of Medicine, South Texas Diabetes and Obesity Institute, University of Texas Rio Grande Valley, Edinburg, TX, United States, [7] Division of General Internal Medicine, Department of Medicine, Albert Einstein College of Medicine, New York City, NY, United States

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Edited by:
Harriet Mayanja-Kizza, Makerere University, Uganda

Reviewed by:
María Teresa Tórtola Fernández, Vall d'Hebron University Hospital, Spain
Wondwossen Amogne Degu, Addis Ababa University, Ethiopia

***Correspondence:**
Juan Ignacio García
juangarcia@txbiomed.org
Blanca I. Restrepo
Blanca.I.Restrepo@uth.tmc.edu
Marcel Yotebieng
marcel.yotebieng@einsteinmed.org
Jordi B. Torrelles
jtorrelles@txbiomed.org

[†]These authors have contributed equally to this work

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Tuberculosis (TB), considered an ancient disease, is still killing one person every 21 seconds. Diagnosis of *Mycobacterium tuberculosis (M.tb)* still has many challenges, especially in low and middle-income countries with high burden disease rates. Over the last two decades, the amount of drug-resistant (DR)-TB cases has been increasing, from mono-resistant (mainly for isoniazid or rifampicin resistance) to extremely drug resistant TB. DR-TB is problematic to diagnose and treat, and thus, needs more resources to manage it. Together with+ TB clinical symptoms, phenotypic and genotypic diagnosis of TB includes a series of tests that can be used on different specimens to determine if a person has TB, as well as if the *M.tb* strain+ causing the disease is drug susceptible or resistant. Here, we review and discuss advantages and disadvantages of phenotypic *vs*. genotypic drug susceptibility testing for DR-TB, advances in TB immunodiagnostics, and propose a call to improve deployable and low-cost TB diagnostic tests to control the DR-TB burden, especially in light of the increase of the global burden of bacterial antimicrobial resistance, and the potentially long term impact of the coronavirus disease 2019 (COVID-19) disruption on TB programs.

Keywords: TB diagnostics, multi-drug resistance, anti-TB drug regimens, active TB, point of care (POC)

INTRODUCTION

The World Health Organization (WHO) estimates that over the next 35 years, without proper surveillance and diagnosis, approximately 75 million people will suffer from drug resistant (DR)-tuberculosis (TB), costing the global economy $16.7 trillion dollars (1, 2). These numbers may fall short due to the projected negative impact of the coronavirus disease 2019 (COVID-19) pandemic on TB control. For decades, the WHO has relied on a simplified and pragmatic approach offering

standardized drug regimens to everyone to treat TB. However, the control and management of the rising burden of DR-TB requires universal access to drug susceptibility testing (DST) and individualized treatment approaches. Currently, in high TB burden countries, there are limited diagnostic options to test for DR-TB. Tests such as the BACTEC™ Mycobacteria Growth Indicator Tubes (MGIT), Xpert or Xpert Ultra ® MTB/RIF, Truenat MTB/MTB Plus/MTB-RIF DX, and Line Probe Assays (LPA), although provide results for DR-TB, are costly and require complex equipment, laboratory infrastructures, biosafety needs, and training for lab technicians. These facts limit their deployment in point of care (POC) settings in low resource communities, where DR-TB cases are rising, specially due to the impact of the COVID-19 pandemic limiting DR-TB testing and treatment (lack of drug supplies), and dedicated personnel (3). Thus, there is a need to develop improved POC diagnostic tests for prompt DR-TB diagnosis and treatment monitoring.

Despite the substantial progress in overall TB control that resulted in a 47% decrease in TB mortality between 1990 and 2015, TB still remains as one of the single infectious diseases associated with high mortality (4). The emergence of several profiles of DR-TB over the past 30 years has further complicated the use of standardized drug treatment regimens. Even pre-COVID-19, estimates indicated that DR-TB will be responsible for more deaths due to antimicrobial resistance than any other single pathogen (5).

Halting the burden of DR-TB will require access to high quality drug susceptibility testing to inform and guide individualized regimens for every TB patient, particularly in high-burden low resource settings. Here, we review the current diagnostic landscape for DR-TB including those in development to assess their suitability and sustainability for DR-TB control based on individualized regimens. Importantly, we explore the possibility of applying known serological test for active TB, as well as to determine if a given patient is responding to treatment. The need for specific *M.tb* and/or host biomarkers to differentiate drug-susceptible (DS) and DR-TB with a simple POC test is also discussed.

UPDATED DR-TB DEFINITIONS

Since the early 1990s, TB prevention and care have been complicated by the growing global burden of rifampicin-resistant (RR) and multidrug-resistant (MDR)-TB. MDR-TB is defined as infection with *M.tb* resistant to at least the first line drugs isoniazid (INH) and rifampicin (RIF) (6). Recently, a WHO expert consultation meeting updated the definitions for extensively-drug resistant (XDR-TB) (7); since January 2022, pre-XDR-TB is defined as TB caused by *M.tb* strains that fulfill the definition of MDR/RR-TB and that are also resistant to any fluoroquinolone, and XDR-TB is defined as TB caused by *M.tb* strains that fulfill the definition of MDR/RR-TB and that are also resistant to any fluoroquinolone and at least one additional Group A drug. The Group A drugs are currently levofloxacin or moxifloxacin, bedaquiline and linezolid; therefore, XDR-TB is MDR/RR-TB that is resistant to a fluoroquinolone and either bedaquiline or linezolid, or both (7). Further, the terms, extremely-drug-resistant TB (XXDR-TB) and total-drug-resistant TB (TDR-TB) were proposed by specific studies to describe the cohort of patients resistant to all tested anti-TB drugs (8–11). However, these terms are not recognized by the WHO as DST is technically challenging and cannot be thoroughly tested since current drug effectiveness against TDR strains are not extensively reported (10). XXDR-TB refers to strains that are resistant to all first and second line anti-TB drugs (8). XXDR resistance, although difficult to treat, is distinct from TDR-TB. TDR-TB is reserved for *M.tb* strains that demonstrate resistance to all available first- and second-line anti-TB drugs, including drugs in the discovery pipeline (9). This drug resistance is thought to be attributed to bacterial chromosomal mutations, inadequate treatments, lengthy drug regimens, patient non-compliance, and presence of other comorbidities, among other reasons (12, 13).

In the last 15 years, while the numbers of deaths due to DS- and DR-TB are estimated to have declined by 20.6% and 28.9%, respectively, deaths due to pre-XDR/XDR-TB have significantly increased by 67.6% (14). Indeed, cases of natural polymorphisms in *M.tb* conferring resistance to the newest anti-TB drug delamanid in drug-naïve patients are reported in countries where this drug had not yet been introduced (15). Similarly, discordant results between laboratories testing the same patient's sample, different patient's samples, and/or between genotypic and phenotypic testing of the same sample, add a layer of complexity to the already difficult task of identifying and properly classifying DR-TB. In this context, in a recent study looking at mortality among people with TB living in various settings across the globe, researchers compared phenotypic or genotypic DS-TB test results obtained from both local and a Switzerland reference laboratories testing identical samples (16). Discordant results were found between local and reference laboratories in about 20% of the samples assessed. Mortality ranged from 6% in people with pan-susceptible TB treated according to WHO guidelines, to 57% in people with DR-TB who went under-wrong treatment because of the discordance between both the local and reference laboratories (16). Interestingly, people with INH monoresistant TB, the most common form of DR-TB globally, had a higher mortality rate compared to MDR-TB patients (16). Thus, in order to reduce TB mortality among DR-TB patients timely access to accurate DST is essential for every TB patient to inform and guide the therapeutic decision.

CURRENT LANDSCAPE FOR UNIVERSAL DST

Phenotypic DST

WHO recommends rapid diagnostics and universal DST (at least RIF resistance testing) for all people experiencing clinical symptoms of active TB, although the current available tools make this goal impossible (17, 18). There are a number of genotypic and phenotypic DST diagnostic tools available for

DR-TB diagnosis; however, their optimal use and their results interpretation are critical for a timely and accurate DR-TB diagnosis to enable an effective patient treatment and care (19).

Among the phenotypic DST used for DR-TB diagnosis, culture, both solid and liquid, is the most commonly used method. Thus, a confirmed *M.tb* specimen is further cultured in solid or liquid media containing the critical concentration of a given anti-TB drug. Lack of *M.tb* growth indicates susceptibility to a given drug, while *M.tb* growth indicates resistance. Culture also provides information on the critical and the minimum inhibitory concentrations (MIC), where MIC is the lowest anti-TB drug concentration capable of inhibiting the growth of a *M.tb* strain.

Phenotypic DST in solid medium is standardized and the most frequently used form of DST in mid and low-income areas endemic for TB (**Table 1**). Löwenstein–Jensen (LJ) slants is the most widely used medium followed by Middlebrook (M) 7H10 or 7H11 agar (20). *M.tb* growth is visualized by the typical rough colonies and cording formations. The indirect proportion method is the most commonly used for solid medium DST using a standardized and two 10-fold diluted dilutions of the inoculum with the anti-TB drug MIC tested (21). Drug resistance is defined when at least 1% of growth is observed at the drug MIC when compared to growth without drug. In many cases, due to the need to obtain enough bacterial growth for DST, specimens are first grown in LJ cultures and only after growth is detected the DST is performed. This considerably delays DST results, which can take 28–42 days or longer to obtain and report.

For phenotypic DST in liquid medium, liquid medium Middlebrook (M)7H9 or M7H10 broths are normally used for mycobacterial growth indicator tube (MGIT) automated *M.tb* culture system [Becton Dickinson and Company (BD) Diagnostic Systems, Sparks, MD, USA]. A specimen is added to the MGIT in the presence of a given concentration of anti-TB drug. Bacterial growth is detected automatically by fluorescence due to oxygen consumption by the presence of *M.tb*, indicating

TABLE 1 | Drug susceptibility tests for implementation in mid and low-income countries with high TB burden.

	TB technology test	Method pirnciple	Cost[#]	Setting to be used	Turnaround time	Complexity	Point-of-care potential	WHO endorsed
Phenotypic DST*	BACTEC 460/960	Liquid culture	$$$	Reference lab	10-42 days	High	No	Yes
	Löwenstein-Jensen	Solid culture	$	Peripheral lab	30-45 days	Moderate	No	Yes
	7H10/7H11 agar	Solid culture	$	Peripheral lab	21-28 days	Low	No	Yes
	1G/2G Color plates	Solid culture	$	Peripheral lab	14-21 days	Low	No	No
Genotypic DST test	GeneXpert MTB/RIF	qPCR	$$$	District lab	<2h	Low	Yes, if availability of GX-Edge or Omni platforms	Yes
	GeneXpert MTB/RIF Ultra	qPCR	$$$	District lab	<2h	Low	Yes, if availability of GX-Edge or Omni platforms	Yes
	GeneXpert MTB/XDR**	qPCR	$$$	District lab	1.5h	Low	Yes, if availability of GX-Edge or Omni platforms	Yes
	TB-LAMP	Loop-mediated isothermal amplification	$$$	Peripheral lab	2h	Low	Yes	Yes
	GenoType MTBDRplus (1st line LPA)	PCR, hybridation	$$	Reference lab	5h	Moderate	No	Yes
	GenoType MTBDRs (2nd line LPA)	PCR, hybridation	$$	Reference lab	5h	Moderate	No	Yes
	FluoroType MTB and FluoroType MTBDR**	PCR, hybridation	$$	Reference lab	2.5h	Moderate	No	Yes
	Genoscholar PZA-TB**	PCR, hybridation	$$	Reference lab	1 day	Moderate		Yes
	Truenat MTB Plus	Micro RT-PCR	$$	Peripheral lab	2h	Low	Yes, onTruelab platform	Yes
	Truenat MTB-Rif Dx	Micro RT-PCR	$$	Peripheral lab	2h	Low	Yes, onTruelab platform	Yes
	Next generation sequencing (NGS)	Gene sequencing (WGS, GWAS)	$$$	Reference lab	5-10 days	High	No	
	Abbott RealTime MTB**	PCR	$$$	Reference lab	11.25h	Moderate	No	Yes
	Abbott RealTime MTB RIF/INH**	PCR	$$$	Reference lab	11.25h	Moderate	No	Yes
	Cobas MTB and cobas MTB-RIF/INH**	PCR	$$$	Reference lab	4.5h	Moderate	No	Yes

**Additional phenotypic assays include, microscopically observed drug susceptibility assay (MODS), and colorimetric redox indicator (CRI).*

***Last recommendations from WHO consolidated guidelines on rapid diagnostics for TB detection, 2021.*

[#]Costs are indicative using the range $-$$$ and includes set up, per test costs, and maintenance needs.

$, Low cost; $$, Medium cost; $$$, High cost.

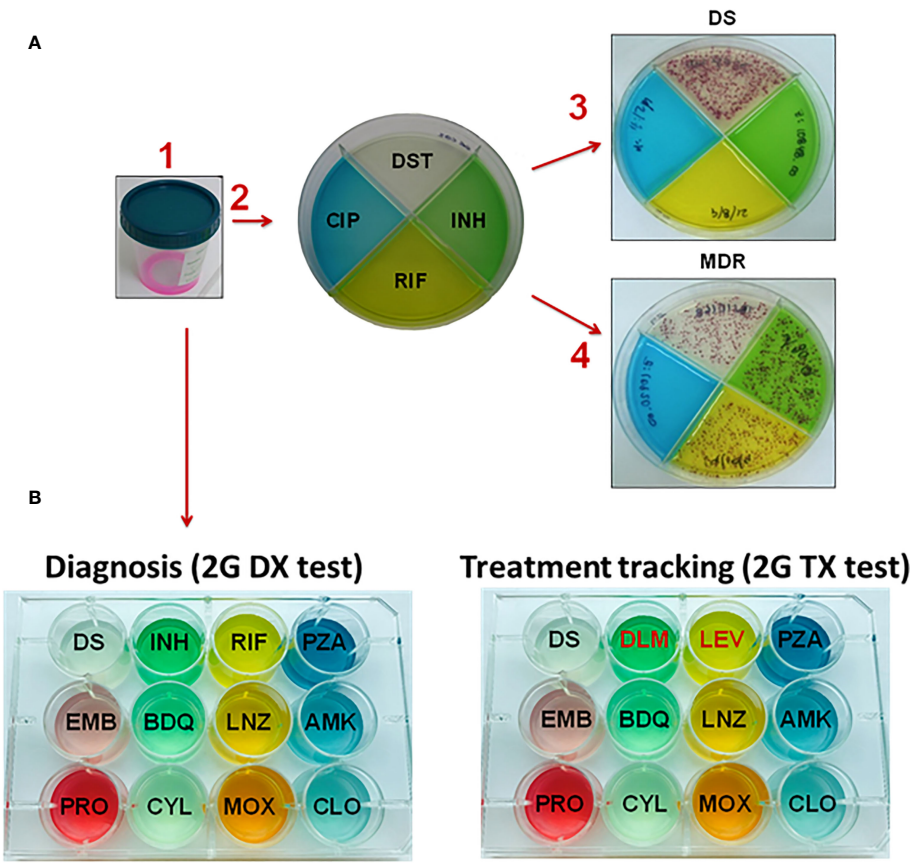

FIGURE 1 | Layout of the 1G and 2G test used to diagnose drug-resistance of *M.tb* infection. **(A)** The 1G test in which four different drugs are contained in each quadrant. A patient's sputum is collected and de-contaminated. Following the arrows, (1) sputum is mixed with decontaminant, (2) the mixture of decontaminant plus sputum (2:1, v/v) is plated into the 1G test. (3) Showing a patient with DS-TB in which colonies are only present in the DS quadrant and not in any of the quadrants with drugs present. (4) Showing a patient with MDR-TB since *M.tb* colonies grow in three of the quadrants, which include no drug (clear quadrant), isoniazid (INH, green quadrant) and rifampicin (RIF, yellow quadrant). **(B)** The 2G test showing the expansion from a quadrant plate (1G test) to 12-wells plate. In this case, the 2G test has 11 different anti-TB drugs that can be used to i) diagnose patients with TB (2G DX test), and ii) track the treatment progression to determine if a subject is responding well or not to the treatment (2G TX test). Subjects with MDR-TB and XDR-TB are by definition INH and RIF resistant; thus the treatment tracking plate has two replacement drugs, in this case DLM and LEV. Abbreviations: DS (drug susceptible); INH (isoniazid), RIF (rifampicin), PZA (pyrazinamide), EMB (ethambutol), BDQ (bedaquiline), LNZ (linezolid), AMK (amikacin), PRO (prothionamide), CYL (cycloserine), MOX (moxifloxacin), and CLO (clofazimine), DLM (delamanid), LEV (levofloxacin).

that *M.tb* is present and it is resistant to the given drug being tested. MGIT DST is quicker than solid media, and it can take up to 14 to obtain results.

For first-line agents (INH/RIF) and some second-line anti-TB drugs [KAN, AMK, ofloxacin (OXO), levofloxacin (LEV)], phenotypic DST is generally consistent, reproducible, and widely used, except for pyrazinamide (PZA), which requires technical expertise to avoid high rates of false positive resistance using MGIT (20). Other anti-TB drugs such as fluoroquinolones [moxifloxacin (MOX), gatifloxacin], cycloserine, capreomycin, ethionamide and prothionamide, as well as re-purposed drugs clofazimine and linezolid are gaining importance to treat DR-TB, and thus, their concentrations for solid DST need to be consistently reevaluated and standardized worldwide. Further, new drugs for MDR-TB treatment such as bedaquiline and delamanid are recommended for use by WHO under specific conditions and may be added to a core MDR-TB regimen (22), and thus their phenotypic DST reevaluation will be also a priority. This is critical as although phenotypic DST is considered by many a step backwards to improve DR-TB testing, there is evidence that show that INH- and RIF resistant TB cases are missed when using genotypic DST, resulting in misleading treatments (23). This is mainly due to the fact that genotypic DST can miss the detection of novel resistance-conferring mutations that are otherwise detected by phenotypic DST (24–29).

In order to improve phenotypic DST and make it more affordable and reliable in mid and low-income countries with high TB burden rates, our group has participated in the field testing of the first generation (1G) Color Plate (1G test) (30), an inexpensive and simple diagnostic that requires minimal training (31–34). The 1G test is a non-commercial test that is based on the

thin-layer agar (TLA) method with an added compound, 2,3 diphenyl-5-(2-thienyl) tetrazolium chloride (STC), that serves as an oxidation-reduction indicator that results in red *M.tb* colonies. This facilitates the detection of colonies isolated from sputum samples to diagnose active TB disease (31–34). Detection of *M.tb* using TLA has been effective for several years, is relatively inexpensive, and only requires an incubator and a 5-10X magnifier glass to confirm diagnosis (30). The 1G test can be used in rural health facilities where access to other molecular TB tests may be limited (34). This DST diagnostic test is used to identify the *M.tb* complex and detection of resistant strains to drugs such INH, RIF, and ciprofloxacin (CIP) or PZA (30, 32, 34).

The 1G test requires minimum training, and has high sensitivity and specificity (32). Due to the STC indicator and special agar composition, *M.tb* growth is accelerated (detection in ~14 days), contaminations minimized, and colonies are visualized as red dots to the naked eye (30, 32, 34). This provides a simple concurrent readout of whether sputum samples are 1) positive for *M.tb* and 2) identification of drug resistance. The 1G test is divided into four quadrants in which one quadrant detects growth of *M.tb* (susceptible quadrant, no drug) and the other three quadrants detect drug resistance to INH, RIF, and CIP (30) (**Figure 1**). Since the 1G test has been an effective diagnostic tool for TB in rural areas such as Malawi and Ethiopia (31, 32, 34), our next step has been to expand the number of drug resistances that we can detect. Thus, we just expanded the 1G test from a quadrant culture-based test to a 12-well culture, which will provide drug resistance evaluation for 11 drugs (**Figure 1B**). This so-called 2nd Generation Color Plate test (2G test) can be used to: i) diagnose primary DS- or DR-TB; and ii) monitor the treatment success of DS- and DR-TB cases. The 1G test can be used only as a diagnostic test for DS-TB and as mono-resistant to INH or RIF, MDR, or pre-XDR); however, it could not track treatment success. The purpose of the 2G test is to gauge for drug resistance and to track the progression of the treatment response. This is particularly important to provide an accurate initial diagnosis and further track and update treatment plans for patients failing their initial treatment regimen. Currently, the 2G test is under field assessment with two types of test: an initial diagnostic test (2G DX), and a treatment progression monitoring test (2G TX) designed to monitor DR-TB patient treatment evolution. Drug composition for both 2G DX and 2G TX is depicted in **Figure 1B**, including the latest anti-TB drugs to treat DR-TB.

Genotypic DST

Genotypic DST detects specific DNA mutations associated with resistance to specific anti-TB drugs in the *M.tb* genome. Genotypic DST has many advantages, including fast results, testing standardization and high throughput. However, the need of funding to support expensive equipment and supplies, a maintenance plan and quality assurance, specialized labs, and an uninterrupted electrical supply, as well as, an excessive cost per test, holds genotypic DST for being implemented globally for the management of DR-TB, especially in mid- and low-income regions with high TB incidence.

The development and implementation of nucleic acid amplification tests (NAATs) have revolutionized the TB and RR/MDR-TB field. As NAATs detect *M.tb* DNA in specimens, their performance correlates directly with the quality of the specimen tested. In the last update for rapid diagnostics for TB detection, WHO endorses three NAAT classes for genotypic DST: low, moderate and high based on the type of technology (automated or hybridization), target conditions (DS-, DR-TB), and complexity for its implementation (equipment, training, infrastructure) (18). Examples of low complexity tests include the Xpert MTB/RIF Ultra or Xpert XDR (Cepheid, Sunnyvale, CA, USA). Moderate complexity automated tests include the Abbott RealTi*m*e MTB and RealTi*m*e MTB RIF/INH (by Abbott, Abbott Park, Illinois, USA), FluoroType MTBDR and FluoroType MTB (by Hain Lifescience, Nehren, Germany), BD MAX^{TM} MDR-TB (by BD, Fraklin Lakes, New Jersey, USA), cobas MTB and cobas MTB-RIF/INH (by Roche, Basel, Switzerland) among others. High complexity tests include hybridization based tests such as the Genoscholar PZA-TB (Nipro Corporation, Tokyo, Japan) (18).

Current molecular testing of the first line drugs INH and/or RIF includes the Xpert and commercial line probe assays (LPAs), such as the Nipro NTM + MDRTB detection kit 2 (Nipro Corporation, Tokyo, Japan) and the MTBDRplus assay (Hain Lifescience, Nehren, Germany). The Xpert assay targets the detection of *M.tb* as well as detection of those mutations that confer RIF resistance. As this drug is rarely resistant by itself, RIF resistance (mutations in the *rpoB* gene) is used as a surrogate to define MDR-TB. The detection of RR *via* Xpert compares well with phenotypic DST methods (35). An advantage of LPAs is that these detect both INH and RIF associated mutations. INH resistance conferring mutations are defined in *inhA* (giving low resistance) and *KatG* (giving high resistance) and these account for ~90% of INH resistance as detected using phenotypic DST (36–39). The other ~10% of INH resistance is related to undetermined mutations in the *M.tb* genome, although mutations on *inhA* promotor and coding regions are reported to drive this resistance (40–43).

As a genotypic DST, in settings that can afford it, Xpert is used for initial diagnostic test for all people presented with TB compatible symptoms, followed by, in some cases, phenotypic DST such as culture. Xpert can be performed using both pulmonary and extrapulmonary specimens. Further, the Xpert Edge is now deployed with the goal of being more user friendly, as well as reducing testing cost and enhance efficiency, with a battery support to reach rural areas in need. Indeed, the Xpert XDR-TB is in development to fit the GeneXpert and GeneXpert Edge platforms to simultaneously detect mutations related to INH, fluoroquinolones, and 2nd line anti-TB agents (18, 44, 45). However, future studies will need to focus on the mechanism(s) of resistance behind the new generation of drugs developed or repurposed such as bedaquiline, delamanid, pretomanid, and linezolid among others, in order to develop novel molecular tests to capture *M.tb* developed resistance to these drugs. Nonetheless, the costs of Xpert technology is prohibited for many high TB burden countries, were cartridge supplies cost depend on owning

or leasing the instrument, varying from ~$10 (own instrument) to $100 (for leased instruments, with variations depending on the country) (46, 47), making many health centers to dismiss its use. Further, patients with previous TB may have residual DNA in sputum making Xpert prone to false–positive results (28, 48) that together with its suboptimal sensitivity in special populations such as children, people living with HIV (PLWH) and extrapulmonary TB patients (49, 50) has undermined its impact in long-term patient outcomes (51, 52). However, this technology is evolving and the possibility of generating a multi-array to diagnose co-infections may make the Xpert more cost-effective in the future. This is already thought for dual TB/COVID-19 testing, especially after the deployment of the Xpert Xpress SARS-CoV-2, which can also minimize the impact of using current Xpert equipment to diagnose only COVID-19 in detriment of TB (44).

The Truenat MTB tests (MTB, MTB plus and MTB-RIF Dx) developed by Molvio diagnostics Pvt. Ltd (Bangalore, India) are chip-based real time micro PCR detection assays for diagnosing TB. These tests can be deployed at peripheral health care settings with minimal infrastructure and depend on battery operated devices that extract, detect and amplify DNA from patient sputum samples for detection of DS- and RR-TB. WHO recommends these assays to be used as a diagnostic test for TB rather than smear microscopy/culture (53). An ongoing multicenter prospective clinical evaluation study in 19 clinical sites, shows good diagnostic accuracy results, despite some evidence of imprecision and inconsistency for sensitivity results and the need of more studies to evaluate the affordability and cost-effectiveness of its implementation (53, 54).

First line LPAs (FL-LPAs) for detection of INH/RIF resistance, as well as second-line LPAs (SL-LPAs) for the detection of resistance to second-line injectable drugs (mutations in the *rrs* and *eis* promoter genes) and fluoroquinolones (mutations in the *gyrA* and *gyrB* genes) are also commercially available (18). WHO recommends the use of commercial LPAs as a rule-out test for XDR-TB. However, caution is recommended because the detection of mutations in the *rrs* and/or eis genes by SL-LPA does not mean that the tested *M.tb* strain is resistant to all the drugs within the second line injectable drug class. Further, although a positive LPA result is reliable in smear positive sputum samples, its diagnostic performance in smear negative sputum samples is low, and thus, adopting LPA does not eliminate the need for phenotypic DST capacity, especially in regions with high DR-TB incidence (18, 55, 56). Another limitation for LPA wide implementation is the need of high-standard molecular facilities to perform the test, which makes its implementation highly challenging in many mid- and low income high TB burden regions (18, 44).

Like the Xpert and LPA, other NAATs are the Gen-Probe MTD test (57) and Pyrosequencing (PSQ) (58). Although there is a push for NAATs becoming a standard diagnosis practice for all presumptive TB cases (59), WHO recommends that NAAT must not replace phenotypic DST such as culture or MGIT. A positive NAAT result is recommended to be supported by other tests such as culture, as well as a negative NAAT result should not be used to definitely exclude TB. Overall, increased use of NAAT in DR-TB diagnosis could decrease the time-to-treatment initiation; however, differential implementation, cost and access to NAAT is a limitation in high TB burden communities.

Although still far away from its routine implementation in the field, next generation sequencing technologies (NGS) (60), such as whole-genome sequencing (WGS) of *M.tb* followed by association with phenotypic resistance using genome-wide association studies (GWAS) are also promising tools for the diagnosis of DR-TB (61, 62). Indeed, GWAS has been used for both, identification of host and *M.tb* traits implicated in the evolution of DR-TB. Thus, host GWAS studies have identified polymorphisms correlated to the heritability of host susceptibility to TB (63), as well as, *M.tb* GWAS studies revealed new resistance genes and regulatory regions implicated in the *M.tb* resistance to 11-anti-TB drugs (64). Some of the advantages of WGS-based diagnostic strategies (65) is the accuracy to diagnose DR-TB (66); however, due to much higher costs compared to traditional microbiological techniques and the need for computing and bioinformatics capabilities, NGS in resource-constrained settings is still far from being implemented (7).

Currently, several novel molecular tests for TB detection are under development at different levels of care: from reference labs to decentralized health care settings to be used as POC tests (18). Although not WHO endorsed yet, the Xpert GX-Edge and Omni, WGS, POC NAATS, and centralized DST can add to the current WHO endorsed LPA, Xpert, TB-LAMP, and Truenat to diagnose DS- and DR-TB, although caution needs to be considered for some of these tests as there is very low certainty of evidence for test accuracy for some of them in determining DR-TB (e.g. Truenat by Molbio) (67–69). For detailed current molecular diagnostic tests in the pipeline (e.g. POC NAATs such as Q-POC from QuantuMDx, indigenous Chinese diagnostics, high-throughput centralized diagnostic test, and next generation sequencing) see the review by MacLean *et al.* elsewhere (44).

TB IMMUNODIAGNOSTIC TESTS

Immunodiagnostics of Latent *M.tb* Infection (LTBI)

A challenge to TB control worldwide is being able to identify the individuals who are going to progress to develop active TB disease upon infection. Targeting these individuals for latent *M.tb* infection (LTBI) treatment can substantially reduce active TB risk. Thus, testing for LTBI is indicated when the risk of developing disease is increased, such as recent contacts of new TB patients, or anyone with a potential exposure that has compromised capacity to contain the infection due to altered immunity (70). These include PLWH, poorly-controlled diabetes, taking immunosuppressive medications (e.g. steroids), young (infants and children) or old age, and other conditions including malnutrition (71, 72). Thus, it is important to identify these individuals, but there is no diagnostic gold standard for

LTBI, and particularly for those with high risk of developing active TB.

Two types of LTBI screening tests are available: the tuberculin skin test (TST) and interferon gamma (IFN-γ) release assays (IGRAs). They represent indirect markers of LTBI based on immunological memory of T lymphocytes to *M.tb* antigens. These tests have an acceptable performance and are widely used in the clinical setting, but have limitations: Neither has a gold standard for confirmation of LTBI, they cannot distinguish LTBI from active TB, nor the various stages of the spectrum of LTBI, and they have a low positive predictive value (about 2-3%) for progression from LTBI to active TB disease (73).

The TST consists of the intradermal injection of mycobacterial purified protein derivative (PPD), with recall immunity inducing a delayed-type hypersensitivity measured by local induration approximately 48 h post-administration (74). Limitations include false positives due to cross-reactivity with non-tuberculous mycobacteria (NTM) and possibly with BCG-vaccinated individuals, particularly if re-vaccinated after infancy and multiple times (72). Specificity is 97% in regions where BCG vaccination is not used, and lowers to about 60% in regions where BCG is applied (75, 76). False negatives occur in patient groups who are immunosuppressed (e.g. HIV, malnutrition) or the elderly due to reduced intradermal immunity (72, 77).

IGRA tests are based on the detection IFN-γ production by peripheral blood lymphocytes in response to specific peptides from *M.tb*, mostly early secretory antigenic target protein (ESAT)-6 and culture filtrate protein (CFP)-10. These peptides are more specific than PPD antigen because they are not encoded in the genomes of most other mycobacteria, with assays reaching >95% specificity. IGRAs are conducted ex-vivo after a blood draw, and hence, only require one office visit. There are two commercial IGRA: The T-SPOT.*TB*® (Oxford Immunotec Ltd, Abingdon, United Kingdom) and the QuantiFERON®, which has had several versions, with the current being the QuantiFERON-Gold Plus (QFT-Plus; Qiagen, Germantown, MD, USA) (74). The T-SPOT.*TB* is an enzyme-linked immunosorbent spot assay, while the QuantiFERON uses an ELISA format. The sensitivity of the T-SPOT.*TB* is generally higher compared to QuantiFERON or TSTs (approximately 90%, 80% and 80%, respectively), but this will vary between study populations. Further, although IGRA is a useful diagnostic method for differentiating TB from NTM diseases, in China for example (78), IGRA shows limited value in this discrimination (79). Further, IGRA does not differentiate *M.tb* from *M. kansassii*, *M. szulgai*, and *M. marinum* because these mycobacterial species also have ESAT-6 and CFP-10 (78, 80). Among the elderly both IGRAs perform similarly, which contrast with the reduced sensitivity of TST in this population (77).

In recent years, the focus has shifted to improving the specificity of immunodiagnostics to identify active TB disease *vs*. LTBI. Certain studies have investigated the diagnosis of active TB using multiplex cytokine and chemokine analysis to improve the sensitivity of IGRAs (81–83). Alternatively, the focus is on depicting *M.tb*-specific T-cell responses *via* flow cytometry measuring both phenotype and function of the T cells (84). Other options used the QuantiFERON-TB Gold In-Tube (QFT-GIT) supernatant in people with and without HIV co-infection, to increase the specificity for active TB diagnosis *vs.* LTBI by looking at differences in multiple host cytokine and chemokine biomarkers assessing multiple unstimulated cytokine/chemokines (IFN-γ, MIP-1β, and TGF-α) coupled with stimulated cytokines (TGF-α and VEGF) (84, 85).

Assessing the activation of *M.tb*-specific T cells is an active field to develop TB immunodiagnosis, for example looking at $CD4^+$ IFN-γ^+ T cells for HLA-DR, CD38, and Ki-67 markers, which shows 100% sensitivity and 95% specificity for active TB (86–88). Phenotypic changes on *M.tb*-specific CD4 T cells are also used as surrogate markers for TB treatment efficacy and can help to discriminate between TB (profile: $CD38^{pos}$, $CD27^{low}$), treated TB ($CD38^{neg}$, $CD27^{low}$), and LTBI ($CD38^{neg}$, $CD27^{high}$) (89). Other studies have looked at *M.tb*-specific TNF producing $CD4^+$ T cells along with the detection of *M.tb*-specific $CD8^+$ T cells and found that, in combination, these immunological assays have a 81.1% sensitivity and 86.5% specificity in the diagnosis of active TB (90–92). Active TB is also significantly associated with an increase in CD27- *M.tb*-specific CD4+ T cells, where evaluating CD27+ CD45RA- CD4+ IFN-γ^+ T cells increases diagnostic accuracy of active TB *vs.* cured TB or LTBI even further (93). Indeed, the combination of different blood biomarkers – namely, CD27, CD45RA, and TNF – within the population of $CD4^+$ IFN-γ^+ T cells provides a good acceptable diagnostic accuracy regarding active TB *vs.* LTBI with 92% sensitivity and 97% specificity while reducing the need to obtain a sputum sample (93). Notably, however, these improvements in immunodiagnostics are not POC and do not allow for the detection of DS- *vs.* DR-TB cases within a population of individuals that have active TB, regardless of their HIV status. As aforementioned, the field is still lacking in POCs to identify DR-TB and provide information about the drug resistance profile, particularly in high TB burden areas where DR-TB is more common.

Interestingly, there are studies in progress to determine when a LTBI case will progress to active TB. An expert consultation was convened by WHO in 2015 to develop target product profiles (TPPs) and an evaluation framework for tests aiming to predict progression from LTBI to active TB (94). In this context, indirect blood PCR-based biomarker tests looking at the expression of host immune response genes to *M.tb* infection are being developed and validated as diagnostic tools that can also predict progression from LTBI to active TB, targeting all forms of TB and patient populations (95). Supporting this, a systematic comparison study of 16 host-derived gene expression signatures (96) found that 7 out of 16 signatures predicted progression from LTBI to active TB disease 6 months prior to sputum conversion (e.g. incipient TB), indicating that some host-response-based diagnostics could be generalizable across diverse patient populations and thus, considered for clinical implementation (97).

Immunodiagnostics of Active TB

One of the key pillars of the End TB strategy is based on early TB diagnosis (17, 98). In this regard, some time ago, a report generated together by WHO and the Foundation for Innovative New Diagnostics (FIND) identified four major TPPs, defining the targets and specifications that new diagnostic TB tests should meet (1): a POC non-sputum test capable of detecting all forms of TB (immunodiagnosis biomarker test); (2) a simple, low-cost POC test performed in clinical/rural settings (e.g. healthcare post) to screen and identify those who need further TB testing (triage test, immunodiagnosis or direct Ag-detection); (3) a POC sputum test to detect pulmonary TB to replace the widely used smear microscopy (the smear-replacement test); and (4) a rapid and efficient DST that can identify those in need of first-line drug treatment (a rapid DST test) (99, 100)

Despite the number of TB diagnostics available, there are still challenges to deploy these tests to urban and rural TB healthcare post and facilities (30, 101). Important parameters to consider in the development of POC diagnostics are, an easily accessible sample, rapid results, high sensitivity and specificity, and cost (less than USD $4 in the location where the test is performed) (101). Currently, available POC diagnostics include the Determine™ lipoarabinomannan (TB-LAM) Ag test (antigen detection), FujiLAM TB test (antigen detection), LIODetect® TB-ST TB Rapid Test developed by LIONEX (antibody detection), and smartphone diagnostics discussed below.

Several POC TB diagnostics are based on the detection of a unique cell envelope component of *M.tb* complex, the mannose-capped lipoarabinomannan (ManLAM) (102). ManLAM is a lipoglycan antigen (Ag) present with a defined role in the survival of *M.tb* during infection (102–104). Specifically, the Determine™ TB-LAM Ag test (Abbott Rapid diagnostics, Abbott Park, IL USA) is a rapid lateral flow Ag-detection POC diagnostic TB test that uses non-invasive samples such as urine to detect undisclosed motifs on the *M.tb* ManLAM structure (104). This diagnostic test is considered to be a 'real' POC test, as it can be used and result read in the field within 25 minutes. This test can be useful to confirm active TB cases, specifically in PLWH, or with sputum smear negative results; however, the test has low sensitivity (52% sensitivity and 98% specificity) (104, 105). To improve upon the sensitivity of this test, our lab demonstrated a simple biochemical step using α-mannosidase to treat the urine samples to cleave the mannose caps on the structure of LAM, providing an increased affinity for LAM antibodies to bind and recognize the epitopes (105). This additional step only added 15 minutes and $0.50 to the cost (final $3.50 per test). The FujiLAM TB test is another novel POC that detects LAM in the urine of patients with clinical symptoms of active TB disease (106). Specifically, the FujiLAM test detects the 5-methyl-thio-*D*-xylofuranose (MTX) motif found on the non-reducing terminal end of the ManLAM structure. The MTX motif reduces cross-reactivity with *M.tb* complex, thus it could be considered a rapid POC TB test that detects positive active TB cases (107, 108). The FujiLAM test has shown a superior diagnostic sensitivity in inpatients with HIV compared to the Determine™ TB-LAM Ag test (70.4% *vs.* 42.3%) (109). The FujiLAM test also shows similar sensitivities (at 75%) in both PLWH and people without HIV infection (110). Furthermore, FujiLAM test shows a substantial higher sensitivity compared to the Determine™ TB-LAM Ag test for detecting extra-pulmonary TB in PLWH (111). Although both the Determine™ TB-LAM Ag and FujiLAM TB tests are rapid POC diagnostics, there is still uncertainty of their sensitivity among HIV negative patients and cannot differentiate between DS- *vs.* DR-TB (112).

Additionally, TB serological tests are used in mid and low income countries to detect the presence of antibodies for TB. One such serological test is the Anda-TB test developed by Anda Biologicals, which looks for the presence of either IgG, IgA, or IgM antibodies specific for the *M.tb* A60 antigen. Meta-analysis of the Anda-TB IgG test revealed a pooled sensitivity of 76% and specificity of 92% in individulas that were acid-fast bacilli (AFB) smear positive, and 59% sensitivity and 91% specificity in AFB smear negative individuals (113). Another TB POC is the LIODetect® TB-ST Rapid Test developed by LIONEX, which detects IgG, IgA and IgM antibodies to *M.tb* antigens in serum, plasma, or whole blood under 20 minutes with 65% sensitivity and 98% specificity (114).

Another aspect of POC diagnostics is the addition of simple adaptors to smartphones for reading results, simplifying the interphase required for TB diagnostics and improving accessibility given their use in daily life, high connectivity and functionality while being portable (115). Smartphones can be equipped with simple adaptors and apps that can be used to capture images for visualization and TB diagnosis (116). Another incentive for smartphones being added to the POC arsenal is that there are project initiatives such as Fair Phone and ARA that can provide low cost manufacturing on a large scale (115). Thus, new technologies and research efforts are imperative, especially in the context of POCs, to provide reliable results in a timely manner and at a low cost (100, 101).

On the light of immunodiagnosis specifically for DR-TB, nothing is developed or is known to be in the discovery pipeline. In this regard, metabolic studies in specimens isolated from DS- and DR-TB patients may bring some light into the discovery of biomarkers that are solely expressed when a person is infected with DR-*M.tb*. However, this seems not plausible. It will be challenging to identify a host biomarker panel to differentiate the drug resistant profile of the infecting *M.tb* strain. Even, if it is doable, a push back from the TB scientific community is expected, as evidences exists that immunodiagnostic tests can be inaccurate and thus, do not improve patient outcomes, and are considered suboptimal tests to be used for pulmonary and extrapulmonary active TB diagnosis in mid and low income countries with high TB burden.

CONCLUSION

Despite recent and encouraging advances towards TB elimination, the ongoing COVID-19 pandemic disruption has undermined these achievements. Cases of *M.tb* infection

reactivation in people recovered from severe COVID-19 are reported (117, 118). The struggle of using recourses for TB diagnosis and care to manage the current demand on COVID-19 is expected to have an impact on TB control (119–121). One of the key aspects to target TB elimination is early TB diagnosis that needs affordable and high-sensitivity POC tests able to diagnose pulmonary and extra pulmonary TB in adults, children, and PLWH (100). To fill current gaps in DS- and DR-TB diagnosis, it is also imperative to develop novel sputum and non-sputum based POC or semi-POC (deployable in rural areas and health-post with minimal resources) diagnostic tests able to characterize drug resistance to improve proper TB care and long term TB treatment outcomes. We will need to consider revisiting phenotypic DST, as genotypic DST, despite its many advantages, has many social-economic barriers, most importantly the high cost that mid and low-income countries with high TB burden cannot afford, even with the assistance of WHO and the good faith of companies developing these technologies. In this context, even with WHO endorsement, national policy needs to be established to ensure test adoption, scale up, and implementation (122). At the end, the cost of supplies, the need of sophisticated technology requiring repeatedly calibrations and staff training; plus, the fact that current genotypic DST cannot identify resistance to current drugs (such as bedaquiline, delamanid and others), and misses the detection of some unknown mutations driving resistance to first line drugs, makes an improved phenotypic DST a need to cover a current global public health demand.

LIMITATIONS

This narrative review is not a systematic review of the literature, and therefore it might unintentionally not include all published papers related to the topic discussed.

AUTHOR CONTRIBUTIONS

All authors listed have made a substantial, direct, and intellectual contribution to the work, and approved it for publication.

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Harriet Mayanja-Kizza,
Makerere University, Uganda

REVIEWED BY
Sara Suliman,
Brigham and Women's Hospital and Harvard Medical School, United States
Nathella Pavan Kumar,
National Institute of Research in Tuberculosis (ICMR), India

*CORRESPONDENCE
Adina Turcu-Stiolica
adina.turcu@gmail.com

†These authors have contributed equally to this work

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Screening performance of C-reactive protein for active pulmonary tuberculosis in HIV-positive patients: A systematic review with a meta-analysis

Andreea-Daniela Meca[1†], Adina Turcu-Stiolica[2*†], Maria Bogdan[1†], Mihaela-Simona Subtirelu[2†], Relu Cocoș[3,4], Bogdan Silviu Ungureanu[5], Beatrice Mahler[4,6] and Catalina-Gabriela Pisoschi[7]

[1]Department of Pharmacology, University of Medicine and Pharmacy of Craiova, Craiova, Romania, [2]Department of Pharmacoeconomics, University of Medicine and Pharmacy of Craiova, Craiova, Romania, [3]Department of Medical Genetics, University of Medicine and Pharmacy "Carol Davila", Bucharest, Romania, [4]Marius Nasta Institute of Pneumology, Bucharest, Romania, [5]Research Center of Gastroenterology and Hepatology, University of Medicine and Pharmacy of Craiova, Craiova, Romania, [6]Pneumology Department (II), University of Medicine and Pharmacy "Carol Davila", Bucharest, Romania, [7]Department of Biochemistry, University of Medicine and Pharmacy of Craiova, Craiova, Romania

Background: Tuberculosis (TB) is the leading infectious cause of mortality worldwide. In the last years, resistant strains of the etiological agent, *Mycobacterium tuberculosis*, have emerged, thus demanding more triage tests to identify active pulmonary TB (PTB) patients and to evaluate their disease severity. Therefore, acute-phase reaction serum tests are required for monitoring TB patients, among WHO symptom screening recommendations. C-reactive protein (CRP) is a non-specific inflammatory biomarker that has been recently proposed for TB screening and can be quantitatively analyzed through cost-effective point-of-care assays. A previous meta-analysis found CRP to be highly sensitive and moderately specific for active PTB with confirmed HIV infection.

Methods: We performed a meta-analysis update of diagnostic tests, pooling sensitivities, and specificities in order to assess the accuracy of CRP as a potential test for the screening of HIV-associated PTB in outpatients. We searched MEDLINE, Web of Science, and SCOPUS for eligible articles before 19 October 2021.

Results: We identified 13 eligible studies with HIV-positive patients with PTB. At a CRP threshold of 10 mg/L, CRP pooled sensitivity was 87% (76%–93%) and pooled specificity was 67% (49%–81%), with an area under the curve (AUC) of 0.858. Using a CRP threshold of 8 mg/L, pooled sensitivity was 82% (72%–89%) and pooled specificity was 82% (67%–92%), with an AUC of 0.879. We found

that CRP has a high sensitivity in the screening of PTB in HIV-positive outpatients, consistent with findings reported previously.

Conclusions: Regardless of pooled specificity, better results were found using the CRP threshold of 8 mg/L as a test screening of PTB, meeting the need of further approaching specific TB diagnostic methods and reducing resource consumption.

KEYWORDS

tuberculosis, HIV-positive, C-reactive protein, screening, sensitivity, specificity

Introduction

The evaluation of tuberculosis (TB) is necessary in order to achieve the World Health Organization's (WHO) TB strategy targets. Although WHO expects a reduction in the epidemic TB incidence rate of 90% by 2035 compared with 2015 (which means less than 10 new cases/100,000 individuals), unfortunately, in 2018, the burden of this infectious disease was still high (1, 2). The total global TB incidence in 2018 was 132 new cases/100,000 individuals (7,253,116 new and relapsed TB notified cases), especially in HIV-positive patients (which represented 64% from total number of cases) (1, 2). In 2020, the global TB incidence was 127 new cases/100,000 population (9,870,000 individuals including 157,817 people diagnosed with drug-resistant TB), which represented one infected person every 3 s (1, 2). Even more, an increase in TB mortality rates was noted in 2020 (as 1.3 million deaths among HIV-negative patients and an additional 214,000 among HIV-positive patients were declared in comparison with 1.2 million and 209,000 declared in 2019, respectively) due to the COVID-19 pandemic, which further involved reduced access to hospitals and pharmacotherapy (3). Even though efforts of improving TB care services have been made in the last years, this major infectious disease remains a worldwide threat, difficult to early diagnose and detect, generating high public transmission rates and mortality (4). Since 2014, it has exceeded HIV, becoming one of the top 10 causes of deaths globally (5). The immediate priority claimed by WHO is to restore access to essential services such as anti-TB treatment, preventive measures, drug-susceptibility testing, and rapid case detection through systematic screening (3).

The performance of adequate laboratory methods in the diagnosis of pulmonary tuberculosis (PTB) depends on many factors. First of all, sputum quality and quantity can have an impact on the yield of TB diagnostic results obtained from the microscopic examination of *Mycobacterium tuberculosis* (*M.tb.*) through the Ziehl–Neelsen method and a culture-based technique (6, 7). On the other hand, *M.tb.* requires an elaborated clinical plan in order to be correctly identified and rapidly eliminated, due to the fact that there is more than one mycobacterial strain that can be detected through microscopic Ziehl–Neelsen examination [also called sputum acid-fast bacilli (AFB) test], but only one generates chronic PTB (8, 9). In other words, sputum microscopy, although fast and inexpensive, is characterized by low sensitivity (61%), therefore involving a low detection rate of *M.tb.* infection (7, 8). A sputum-smear negative and/or negative culture does not always exclude TB diagnosis and may lead to wrong TB management (7–9); this accelerated the use of Xpert Gene MTB/RIF automated rapid molecular assay, which is less sensitive than culture (92%), as a more sensitive method than sputum-smear microscopy for fast identification of PTB as well as rapid assessment of rifampicin susceptibility (6, 10, 11). However, available diagnostic tests and mycobacterial cultures are rather time-consuming compared to point-of-care tests and also require both logistical measures and experienced personnel in order to properly diagnose TB (12, 13). Although GeneXpert MTB/RIF assay is preferred as a diagnostic tool in HIV-positive individuals due to higher sensitivity compared to smear microscopy and faster results compared to mycobacterial culture (14), it imposes various demands such as constant connection to electricity, proper temperature flow, and dedicated personnel to ensure installation functionality in comparison with rapid serological assays (13, 15). Even more, following WHO recommendations to maximize case findings, preclinical evaluation is also based on symptom screening (WHO 4-SS: the presence of at least one of the following in the last 30 days: cough, fever, night sweats, or weight loss), characterized by high sensitivity, but reduced specificity, hence low effectiveness in evaluating TB (8, 11, 16). Cicacci et al. draw attention that screening HIV-positive patients through the WHO-4-SS method may lead to more than 22% of missed TB cases (13), as a significant proportion of *M.tb.*-infected individuals may be asymptomatic (17). Consistently, the specificity of the WHO-4-SS screening method for PTB in HIV-positive patients is approximately 70%, challenging already resource-constrained countries (18). Gersh et al.

assessed 0% sensitivity, 87% specificity, and 99% negative predictive value of the WHO-4-SS algorithm, concomitant with 20% sensitivity, 99% specificity, and 99% negative predictive value of the Xpert Gene test for identifying TB in 383 HIV-infected individuals (19). Therefore, the WHO-4-SS screening tool is considered partially effective in HIV-infected patients (17). The most recent point-of-care available triage test detects lipoarabinomannan [lateral flow urine lipoarabinomannan assay (LF-LAM)] in individuals with active TB, as lipoarabinomannan represents an essential lipopolysaccharide for mycobacterial cellular wall (17). LF-LAM has multiple advantages: cost-effectiveness, involves a simple procedure, does not require special equipment, provides rapid results, and high specificity in HIV-infected patients (20). However, LF-LAM presents low sensitivity (56% in patients with CD4 count ≤ 100 cells/µl and 26% in patients with CD4 count > 100 cells/µl), and its specificity decreases proportionally with higher CD4 counts in severe HIV infection (13, 20). Nevertheless, increased sensitivity values for a screening test would rule out TB in non-infected individuals and would also limit the use of more expensive confirmatory tests, as the ones mentioned above, in patients exposed to a higher risk of *M.tb.* infection (thus with increased specificity) (10, 11, 14). Therefore, in order to control TB burden, rapid and reliable screening strategies are imperiously necessary, especially in African regions with an increasing number of HIV-positive patients in the last years (13).

Initiation of anti-TB pharmacotherapy should be approached in suspected but unconfirmed cases as treatment delays may increase transmission burden and may lead to poorer outcomes, especially in patients with immunosuppression (21). In case of HIV infection—the most important risk factor in developing active TB (22)—as soon as antiretroviral therapy is administered, the mortality rate decreases and the prognosis is improved (3, 21, 23). This particular need of early bacterial identification is increased by low-adherence antituberculotic regimens: first-line agents represented by isoniazid, rifampicin, pyrazinamide, ethambutol, and streptomycin (mostly used in primary active TB), and second-line agents (mostly used in resistant bacilli strains) (5, 24–26). First-line regimens are recommended as a directly observed therapy (DOT) in drug-susceptible TB in individuals residing in settings with a low proportion of resistant strains for 6 months as follows: intensive phase (rapid mycobacterial reduction) when agents are administered daily for 2 months and continuation phase (sterilization phase with isoniazid and rifampicin given thrice every week) for the next 4 months (21, 24, 27). In case of isoniazid or rifampicin resistance, an expanded second-line regimen is recommended for more than 12 months, which includes a fluroquinolone (levofloxacin or moxifloxacin) or an injectable agent (such as amikacin, kanamycin, or capreomycin), repurposed drugs (clofazimine or linezolid), or the most recent approved agents (bedaquiline or delamanid) (23, 27). Moreover, continuation phases can be expanded in case of extrapulmonary TB; however, the regimens are similar to the ones used in PTB (27). Recent growth in both multi-drug-resistant TB forms (MDR-TB—due to strains resistant to rifampicin and isoniazid) and extensively drug-resistant TB forms (XDR-TB—due to additional resistance to second-line agents) is caused by the improper use of antibiotics and difficult diagnosis procedures that require even more time than primary active TB, thus urging the necessity to rapidly and specifically diagnose bacterial TB strains (6, 28, 29).

The particularity of PTB lies in the immunological fight between *M.tb.* and the host, based on the interaction of bacterial strains and inflammatory biomarkers released by macrophages, monocytes, neutrophils, and lymphocytes (30–34). C-reactive protein (CRP) is also a non-specific biomarker in TB, with highly increased plasmatic concentrations, due to sputum bacillary load and severity of inflammation (30, 35). Human liver CRP production usually forgoes clinical symptoms (30, 31). CRP can be measured semi-quantitatively using capillary blood or quantitatively from either venous or capillary blood through different immunoturbidimetric methods and rapid cost-effective quantified point-of-care tests (POC-CRP assays) that provide results in less than 5 min (31, 36–38). Moreover, POC-CRP tests are easily interpretable by specialists and available worldwide in comparison with reference diagnosis standards (12). On the other hand, in order to correctly implement POC-CRP tests, the program requires a valid quality method, trained certified applicants, and continuous internal control based on distributors' manuals (38). Although CRP is a non-specific biomarker in inflammatory diseases, researchers have noted elevated levels associated with other bacterial infections, burns, traumas, rheumatic diseases, and various carcinomas and metastatic stages in lung, breast, digestive, hepatic, and ovarian tissues (39). More recently, CRP/albumin ratios have been proven to be a powerful mortality prognosis marker in hypertensive COVID-19 adults (40), and systematic research should be performed in order to assess the screening and diagnostic accuracy of this serologic marker.

In the past years, different types of studies have been published (retrospective, comparative, multi-center, and clinical trials) that claim the use of CRP as a TB screening test for TB (both pulmonary and extra-pulmonary), in various ethnicities, with several comorbid pathologies (12, 41–45). This increased interest in CRP research has prompted us to evaluate through statistical analysis if CRP is an adequate screening tool, as a rule-out test. Recent literature has shown that TB screening could be intensified and improved by using plasmatic CRP concentrations, especially in low-income countries due to the cost-effectiveness of this biomarker analysis (11, 41). Nevertheless, CRP has also been proposed as a solid candidate for TB screening in HIV-positive patients, providing prognostic values and leading to a more productive disease management (37, 46). Shapiro et al. also underlined the

importance of CRP as a discrimination factor between culture-positive and culture-negative specimens (47). Moreover, CRP proved higher accuracy and specificity when evaluated as a TB case-finding test in comparison with WHO-4-SS (12, 47). Although CRP does not identify drug resistance, its potential clinical relevance as a screening test in PTB patients and as a reliable tool in monitoring treatment outcomes justifies the concept of our study (48).

The objective of this study is to determine the accuracy of the use of CRP as a screening biomarker for TB in adults with HIV infection. Our further question refers to the clinical cutoff point(s) of CRP that could indicate a significant inflammation and predicts the presence of PTB in HIV-infected patients. A previous meta-analysis found CRP as a considerable promising tool to ease systematic screening for active TB (49). Since this previous meta-analysis, new studies have been published. WHO promotes intensified TB case identification in HIV-positive adults by WHO 4-SS; thus, we investigated whether rapid CRP tests are more valuable than WHO 4-SS (8, 49, 50). In order to determine the pooled sensitivity and specificity of the CRP test for PTB in outpatients with HIV infection, we performed a meta-analysis update with other subgroups. We planned to conduct a subgroup analysis for CRP cut-points where sufficient data were available.

Methods

Search strategy

This meta-analysis was conducted in accordance with the PRISMA (Preferred Reporting Items for Systematic Reviews and Meta-Analyses) statement. We searched MEDLINE, Web of Science, and SCOPUS, until 19 October 2021, following terms ("tuberculosis" AND "C-reactive protein") OR ("tuberculosis" AND "CRP" AND " screening test" AND "diagnosis"). The study identification also included manual search, with the screening of the citations of the relevant studies. Two review authors (A-D.M. and M-S.S.) independently extracted data using Excel to determine potentially eligible studies. The disagreements were resolved through discussion and, if necessary, consulting a third review author (A.T-S.).

Study selection

Inclusion criteria to identify studies that directly address the research question were carefully defined: patients were limited to ambulatory patients because hospitalized patients may have different acute inflammatory conditions, other than HIV infection conclusive for our study that could influence the CRP level. The inclusion criteria were as follows: (a) randomized clinical trials investigating the CRP in HIV-positive active PTB in adults; (b) studies including HIV-positive patients with symptoms or reactivated manifestations of PTB; (c) studies including HIV-positive patients who have not previously been on antitubercular treatment; (d) studies consisting of original articles, peer-reviewed with randomized controlled trials that evaluated the use of CRP, by examining sensitivity and specificity; (e) studies written in English; and (f) studies including mycobacterial reference standard or/and a composite reference standard diagnosis. Eligible studies in which CRP was measured through quantitative laboratory-based and POC assays were also considered. We only included studies that reported data comparing the index test(s) to an acceptable reference standard from which we could extract true positive (TP), true negative (TN), false positive (FP), and false negative (FN) values. The included studies were selected after reviewing the abstract and full text for eligibility.

We included all published manuscripts that primarily assessed CRP levels marking the presence of PTB and also the gold standard diagnosis criteria for TB. Studies that mentioned GeneXpert MTB/RIF assay or WHO score, sustained by radiographic evidence as diagnosis methods for PTB, were also considered for analysis.

We excluded studies that (a) measured CRP through non-quantitative methods or not measured CRP; (b) lacked CRP cutoff values; (c) discussed comorbid inflammatory conditions in patients without HIV condition; (d) diagnosed TB through methods based on inadequate standard reference; (e) included patients with extrapulmonary TB or other pulmonary infections determined by a different strain of mycobacteria; (f) included children; (g) were written in a language other than English; and (h) contained data insufficient to easily distinguish between TP and TN cases. If we needed more information (for example, TP, TN, FP, and FN at 8 mg/L threshold for CRP), we contacted primary study authors for it. The target condition was active PTB in HIV-infected patients; thus, we excluded the studies that also involved patients with extrapulmonary TB that cannot be separated.

Data extraction and risk of bias assessment

We appraised the quality of studies using the Quality Assessment of Diagnostic Accuracy Studies-2 (QUADAS-2) tool, which consists of four domains: patient selection, index test, reference standard, and flow and timing (differential verification of TB status for study participants). All these domains were assessed for risk and bias.

Statistical analyses

We performed this meta-analysis to estimate pooled sensitivity and specificity using a bivariate random-effects

model and a Bayesian approach. Random-effects models were chosen to describe the variability in test accuracy across studies.

The TP, FP, TN, and FN values were extracted from the included studies or the studies' correspondence authors were contacted to provide us with this information. We presented individual study results graphically in forest plots, by plotting the estimates of sensitivity and specificity [95% confidence interval (CI)].

Exploratory analyses were undertaken in Review Manager 5 (RevMan 5), and we used R for the definitive analyses. The R packages INLA and mada were used for the meta-analysis of diagnostic accuracy. The bivariate model provided a summary receiver operating characteristic (SROC) curve that integrated receiver operator characteristic curves of primary studies. We calculated area under the curve (AUC) and partial AUC for every SROC plot.

We grouped the studies evaluating CRP by the threshold of 8 mg/L and 10 mg/L. Mycobacterial culture (solid or liquid) or composite reference standards or bacterial microscopic examination through the Ziehl–Neelsen method and fluorescent method was used as reference standard. We investigated the key parameters of summary ROC curves and summary sensitivity–specificity points.

Heterogeneity was investigated through visual examination of forest plots and ROC plots of the raw data. Descriptive statistics included the pooled sensitivity, pooled specificity of the studies, their diagnostic odds ratio (DOR, a measure of the effectiveness of the diagnostic test; higher DOS indicates better test performance), Higgins I^2 (assess the consistency of the results of studies in meta-analysis: a value of 0% indicates no observed heterogeneity, and larger values show increasing heterogeneity), and Cochran's Q statistic. We performed χ^2 test to assess heterogeneity of sensitivities and specificities, the null hypothesis being, in both cases, that all are equal for all the studies. The significance level was 0.05.

Sensitivity analyses were performed by limiting inclusion in the meta-analysis to the studies, for example, that scored "yes" for the QUADAS-2 question "Did the study avoid inappropriate exclusions?", which expresses a low risk of bias for participant selection, or, another example, the studies that scored "yes" for the QUADAS-2 question "Is the reference standard likely to correctly classify the target condition?", which leads to a low risk of bias for the reference standard.

Results

Search results

We identified 13 studies that met the inclusion criteria of our present study (eight new studies since the previous review). Figure 1 shows the flow of studies in the review, with the steps of the study selection process in a PRISMA diagram.

All included studies were performed in countries with a high TB/HIV burden, classified as low-income or middle-income countries (World Bank 2020) as described in Table 1 with the other characteristics of included studies.

Bias assessment

The risk of bias and applicability concerns is shown in Figure 2. Most studies had a low risk of bias.

We found an unclear risk of bias in patient selection for Samuels (2021) (14), a retrospective case–control study with no details on how the allocation list was concealed, and Wilson (2006) (51), where it was unclear if patients' randomization was performed. Olson (2019) (16) and Wilson (2011) (52) were considered at high risk of bias selection because the random sequence generation was not included into the patients' selection.

Yoon (2014) (37) was considered to have a high risk of bias for reference standard because the reference standard, its conduct, or its interpretation could have introduced bias and also raised concerns regarding the applicability of the reference standard. In Wilson (2006) (51), it was unclear if the reference standard results were interpreted without knowledge of the results of the index tests.

Samuels (2021) (14) did not clearly explain if the patients received the same reference standard. Wilson (2011) (52) introduced a risk of bias through flow and timing because not all patients were included in the analysis; some of them were not able to attend for regular review.

High applicability concerns were raised for patient selection because the included patients and setting could not match the review question in the case of three studies (50–52).

The pooled results

Thirteen studies were included in the meta-analysis that evaluated CRP for PTB among HIV outpatients (14, 16, 18, 19, 41, 46, 47, 49–54). The studies provided data for 4,355 HIV-positive adults, including 891 (20%) with PTB.

For the CRP threshold of 10 mg/L, sensitivity estimates range between 20% and 98%, and specificity estimates range between 26% and 96%. Twelve studies including 3,751 patients were included in the diagnostic meta-analysis.

The forest plot and SROC curve of CRP sensitivity and specificity for PTB for studies among HIV-positive patients, using the CRP threshold of 10 mg/L, are given in Figure 3. The dotted blue curve represents the 95% confidence region and the dotted closed curve represents the 95% prediction region.

The pooled sensitivity was 87% (95% CI: 76%–93%) and the pooled specificity was 67% (95% CI: 49–81%), test for heterogeneity I^2 = 29.49%, Q = 12.77, p = 0.174, and DOR =

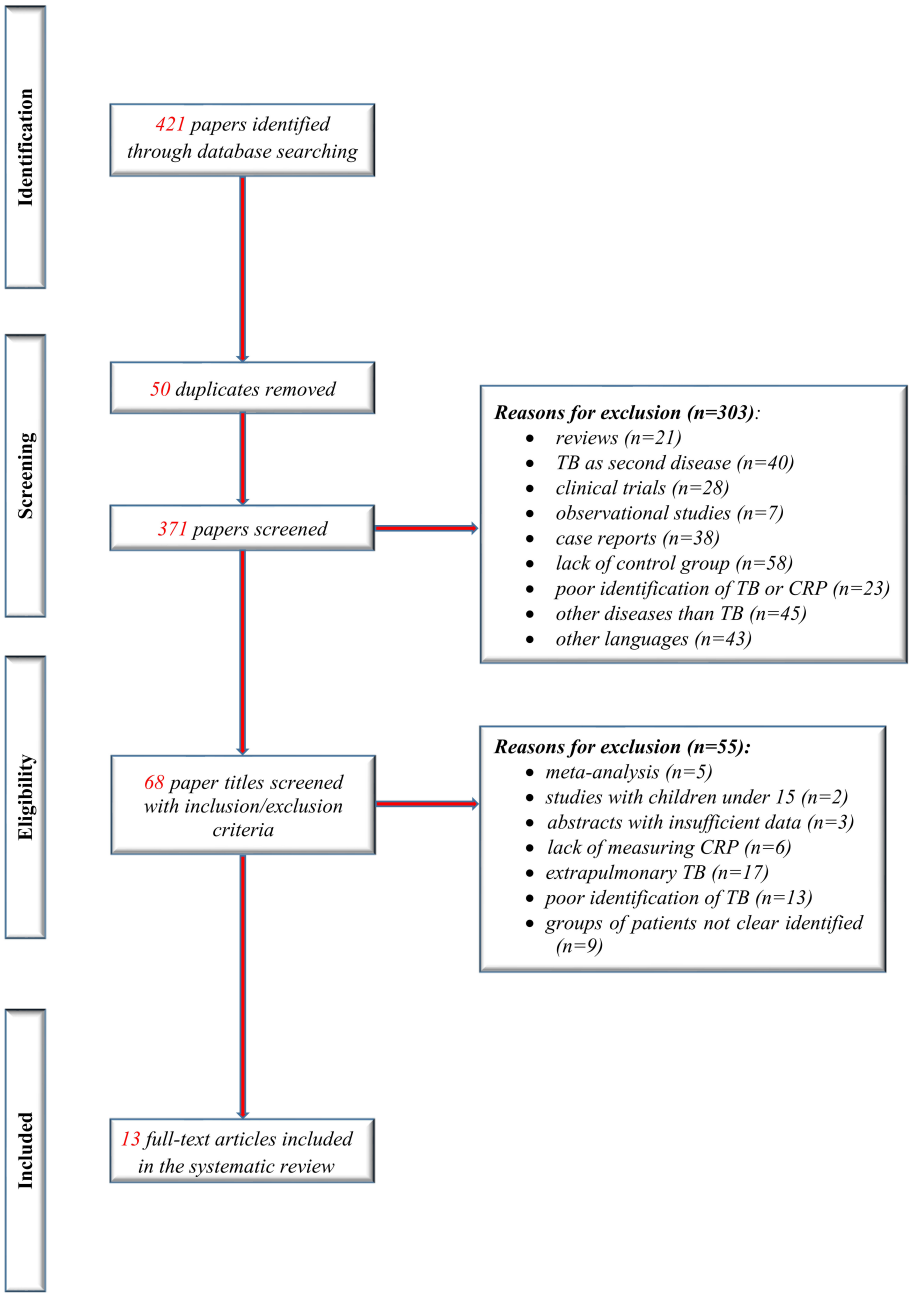

FIGURE 1
Study flow PRISMA diagram.

13.26. The χ^2 test ($p > 0.05$) and $I^2 < 50\%$ suggested non-significant heterogeneity of sensitivities and specificities, so we used fixed-effects meta-analysis. AUC and partial AUC were 0.858 and 0.841, respectively.

For the CRP threshold of 8 mg/L, sensitivity estimates range between 40% and 94%, and specificity estimates range between 49% and 96%. Seven studies including 3,205 patients were included in the diagnostic meta-analysis (14, 16, 19, 41, 47, 49, 54). The forest plot and SROC curve of CRP sensitivity and specificity for PTB for studies among HIV-positive patients, using the CRP threshold of 8 mg/L, are presented in Figure 4.

The pooled sensitivity was 82% (95% CI: 72%–89%) and the pooled specificity was 82% (95% CI: 67%–92%), test for heterogeneity $I^2 = 0\%$, $Q = 4.95$, $p = 0.55$, and DOR = 19.12 (95% CI: 6.5–56.1). The χ^2 test ($p > 0.05$) and $I^2 < 50\%$ suggested non-significant heterogeneity of sensitivities and specificities, so

TABLE 1 Characteristics of included studies.

Study (name, year)	Country	Number, type of patients and median age	Methods of TB screening and diagnostic (*reference standard diagnostic test)	CRP dosing method (assay kit/analyzer type)
Ciccaci 2019 (41)	Mozambique, South Africa	*n* = 143 (21 HIV-positive/Xpert-positive + 122 HIV-positive/Xpert-negative), median age = 36	WHO 4-SS + *Xpert Gene MTB/RIF (Assay system, Cepheid, Sunnyvale, CA, USA)	Measured before anti-TB treatment through enzyme-linked immunosorbent assay—Human CRP ELISA—kit (Arigo Biolaboratories Corporation, Hsinchu City, Taiwan)
Drain 2014 (50)	KwaZulu-Natal, South Africa	*n* = 93 (45 HIV-positive/TB-positive—from which 37 were culture-positive and 8 were SSM-positive—and 48 HIV-positive/TB-negative), median age = 35	*Culture (both liquid BACTEC TB 960 systems and solid) + SSM (both Ziehl–Neelsen and auramine fluorescent staining) + WHO 4-SS + chest radiography evaluation	Measured before anti-TB treatment through both immunoturbidometric assay with Dimension RXL analyzer from Dade-Behring (Deerfield, IL, USA—laboratory-based high-sensitivity method) and immunoassay POC (NycoCard CRP test—Axis-Shield, Oslo, Norway)
Lawn 2013 (46)	Cape Town, South Africa	*n* = 496 (81 HIV-positive/culture-positive + 415 HIV-positive/culture-negative), median age = 33.6	SSM (fluorescent microscopy) + *culture (liquid BACTEC MGIT) + WHO 4-SS + Xpert Gene MTB/RIF + chest radiography evaluation	Measured before anti-TB treatment through enzyme-linked immunosorbent assay—Quantikine (R&D Systems Inc., Minneapolis, MN, USA)
Olsson 2019 (16)	Ethiopia, East Africa	*n* = 260 (130 HIV-positive/PTB-positive + 130 HIV-positive/TB-negative), median age = 33.6	*SSM + *culture (liquid) + WHO 4-SS + *Xpert Gene MTB/RIF (Cepheid, Sunnyvale, CA)	Measured before anti-TB treatment through immunoturbidometric assay (Bio-Rad Laboratories, Hercules, CA—Bio-Plex 200 reader) and Magnetic Luminex Assay (R&D Systems Inc., Minneapolis, MN)
Shapiro 2018 (47)	Durban, South Africa	*n* = 425 (42 HIV-positive/PTB culture-positive + 383 HIV-positive/PTB culture-negative), median age = 32	*Culture (liquid BACTEC MGIT systems) + WHO 4-SS + SSM + Xpert MTB/RIF + chest radiography evaluation	Measured before anti-TB treatment through immunoturbidometric assay through Roche Integra analyzer (Mannheim, Germany)
Wilson 2006 (51)	South Africa	*n* = 130 (105 HIV-positive/TB culture-positive or histological features and detection of any mycobacterial specimen and 25 HIV-positive/possible TB with anti-TB-treatment response), median age = 34.4	SSM (auramine-rhodamine fluorescent microscopy) + *culture (liquid BACTEC MGIT) + chest radiography evaluation	Measured before anti-TB treatment and also assessed at weeks 2, 4, and 8 after anti-TB treatment through immunoturbidometric assay (Beckman Coulter CX7)
Wilson 2011 (52)	KwaZulu-Natal, South Africa	*n* = 200 (112 HIV-positive/TB culture-positive and 88 HIV-positive/extraPTB or both PTB and extraPTB), median age = 34.4	*SSM (fluorescent microscopy) + *culture (liquid BACTEC MGIT) + WHO 4-SS + chest radiography evaluation	Measured before anti-TB treatment or after maximum one week of anti-TB treatment through immunoturbidometric assay (Olympus AU640 and Dade Dimension RXL)
Yoon 2014 (37)	Mbarara, Uganda	*n* = 201 (5 HIV-positive/PTB-positive and 196 HIV-positive/PTB-negative), median age = 33	*SSM + *culture + WHO 4-SS	Measured before anti-TB treatment through immunoassay point-of-care (iCHROMA POC-CRP Reader, BodiTech Med Inc., South Korea)
Yoon 2017 (49)	Kampal, Uganda	*n* = 1,177 (163 HIV-positive/PTB culture-positive, from which 84 PTB also confirmed with Xpert Gene MTB/RIF positive and 1,014 HIV-positive/PTB-negative), median age not mentioned	SSM (Capilia TB, TAUNS, Japan or MPT64 assay, Standard Diagnostics, South Korea) + *culture (solid Löwenstein-Jensen and/or liquid BACTEC MGIT 960) + WHO 4-SS + Xpert Gene MTB/RIF (Cepheid USA)	Measured before anti-TB treatment through immunoassay point-of-care (iCHROMA POC-CRP Reader, BodiTech Med Inc., South Korea)
Boyles 2020 (18)	Johannesburg, South Africa	*n* = 207 (75 HIV-positive/TB-positive + 132 HIV-positive/TB-negative), median age = 36	WHO-4-SS + SSM + *culture (liquid–mycobacterial growth indicator tube, MGIT BACTEC 960 TB System) + Xpert MTB/RIF Ultra (Ultra)	Measured before anti-TB treatment through point-of-care method (Abbot Afinion AS100 analyzer)
Gersh 2021 (19)	Western Kenya	*n* = 383 (5 HIV-positive/TB culture-positive + 378 HIV-positive/TB-negative), median age = 37	WHO-4-SS + SSM (fluorescence microscopy and AFB) + *culture (commercial broth method, MGIT Manual Mycobacterial Growth System, Becton-Dickinson, Franklin Lakes, NJ) + Xpert Gene MTB/RIF (Xpert, Cepheid, Sunnyvale, CA)	Measured before receiving anti-TB treatment, using a high-sensitivity assay (Cobas Integra 400 Plus (Roche Diagnostics, Rotkreuz, Switzerland)

(Continued)

TABLE 1 Continued

Study (name, year)	Country	Number, type of patients and median age	Methods of TB screening and diagnostic (*reference standard diagnostic test)	CRP dosing method (assay kit/analyzer type)
Mwebe 2021 (53)	Kampala, Uganda	*n* = 605 (103 HIV-positive/TB-positive + 502 HIV-positive/TB-negative—confirmed with TB if at least one sputum culture was positive), median age = 34	Xpert Gene MTB/RIF (Cepheid USA) + *liquid mycobacterial culture (BACTEC MGIT 960) + WHO-4-SS	Measured before anti-TB treatment through standard point-of-care assay from capillary blood (iCHROMA POC-CRP Reader, BodiTech Med Inc., South Korea)
Samuels 2021 (14)	South Africa, Cambodia, Peru, Georgia, Vietnam	*n* = 765 (111 HIV-positive/TB culture-positive + 274 HIV-negative/TB culture-positive + 102 HIV-positive/TB-negative + 253 HIV-negative/TB-negative + 6 unknown HIV status/TB-positive + 19 unknown HIV status/TB-negative), median age = 36	SSM (fluorescence microscopy with auramine staining) + *culture (liquid MGIT Becton Dickinson, Franklin Lakes, USA + solid Lowenstein-Jensen medium) + Xpert Gene MTB/RIF (Cepheid, Sunnyvale, USA)	Measured before anti-TB treatment through a latex immunoassay (Multigent CRP Vario assay on Abbott Architect C8000)

TB, tuberculosis; CRP, C-reactive protein; WHO-4SS (4-symptoms screening): symptom screen positivity is defined by the presence of any current cough, fever, night sweats, or weight loss in the previous 30 days; SSM (sputum-smear microscopy): identification of AFB (acid-fast bacilli) through the Ziehl–Neelson method or the auramine fluorescent method; culture: can be realized through solid medium (Löwenstein–Jensen) and/or the liquid BACTEC MGIT (Mycobacterial Growth Indicator Tube) 960 system.

we used fixed-effects meta-analysis. AUC and partial AUC were 0.879 and 0.833, respectively.

Discussion

This meta-analysis updates the current literature, including 13 studies, on the accuracy of the CRP screening test for PTB in HIV-positive adults (14, 16, 18, 19, 41, 46, 47, 49–54). The pooled estimates for sensitivity and specificity did not remain similar using a CRP cut-point of 10 mg/L, compared to the current review (pooled sensitivity = 87% and pooled specificity = 67%) and prior review (pooled sensitivity = 82% and pooled specificity = 82%), respectively (48).

We chose to evaluate the accuracy of the CRP screening test separately for studies that present the results for the threshold of 10 mg/L vs. 8 mg/L because the SROC curves do not estimate with respect to the identification of points on the curve that show a particular threshold. The pooled sensitivity was 86% in the case of the 10 mg/L threshold and 81% in the case of the 8 mg/L threshold. Better pooled specificity was found in the case of the 8 mg/L threshold: 88% vs. 73% in the case of the 10 mg/L threshold.

As WHO recommends, people infected with HIV or living with HIV should be systematically screened for active TB through WHO-4SS assessment or chest radiography evaluation as a second screen test (8, 55, 56). Further clinical diagnostics is established by different algorithms: mycobacterial culture, SSM, and Xpert MTB/RIF test (8, 56). Although culture is the gold standard for TB diagnosis, it is not usually approached as an initial test, due to the longer time required for results (2 to 6 weeks) (8, 56). Thus, in poor-resource and HIV high-prevalence areas, diagnostic algorithms include SSM and Xpert MTB/RIF that provide final results in less than 24 h (8, 56). Even more, a good TB diagnostic test must have at least 90% sensitivity and 70% specificity, and CRP has been proven to be the closest parameter to meet WHO recommendations (14, 53, 56). Deficiency in screening strategies could lead to delayed TB diagnosis or misdiagnosis, higher rates of transmission and mortality, with disastrous financial consequences (8, 56). Various clinical algorithms have been developed and assessed for management of TB; however, these present shortcomings in HIV-positive patients (42, 45). Recent studies suggested that CRP presents higher specificity values and a better performance for the identification of active PTB than WHO symptom screening strategies (16, 46, 55). The meta-analysis conducted by Yoon and collaborators also proved high values (93%) of sensitivity and moderate values of specificity (63%) for CRP as a screening method among individuals with PTB-specific symptoms (49). Samuels et al. included both cutoff points for CRP and obtained even better values for sensitivity and specificity compared with culture diagnostic methods as CRP concentrations decreased: 91.9% sensitivity and 52.9% specificity for a cutoff point of 10 mg/L, and 93.7% sensitivity and 49% specificity for a cutoff point of 8 mg/L (14). Mwebe et al. obtained 81% sensitivity and 71% specificity for CRP diagnostic accuracy when compared to culture methods and confirmed the utility of CRP as a TB screening tool for HIV-positive individuals (53). The maximum value for CRP sensitivity extracted from the included studies in our meta-analysis was 95% at a cutoff point of 10 mg/L, but correlated with 26% specificity compared to sputum tests; the authors characterized CRP as an important predictor for TB (18). Researchers also mentioned that CRP became less specific while increasing sensitivity values proportionally with a larger number of symptoms: 93.5% sensitivity and 37% specificity for CRP > 8 mg/L, and 92.5% sensitivity and 41.4% specificity for CRP > 10 mg/L (14). In accordance with WHO recommendations, when CRP is used as a screening test and provides positive results,

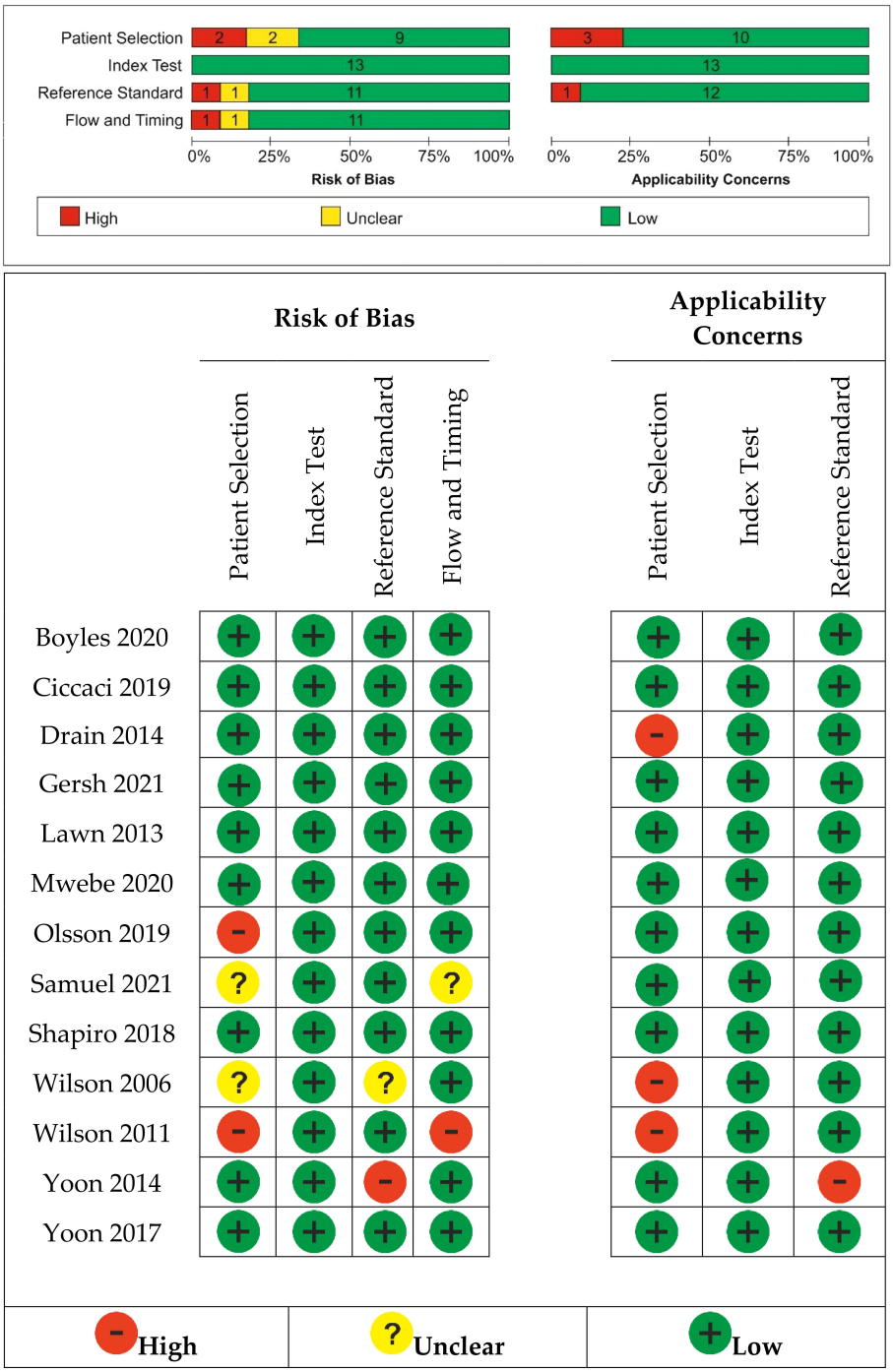

FIGURE 2
Risk of bias and applicability concerns summary: review authors' judgments about each study.

individuals should undergo evaluation through specific diagnostic methods (14). CRP sensitivity reached higher values than 90% in cases of TB diagnosed through Xpert Gene test; therefore, CRP usage could provide cost-effectiveness as it would reduce about 40% the need to use Xpert Gene as a diagnostic method (14). Moreover, the excellent sensitivity of CRP appeared in HIV-positive individuals, but with possible insufficient specificity, raising the need to adjust even more cutoff points for CRP in order to improve screening performance (14, 18). In line with this,

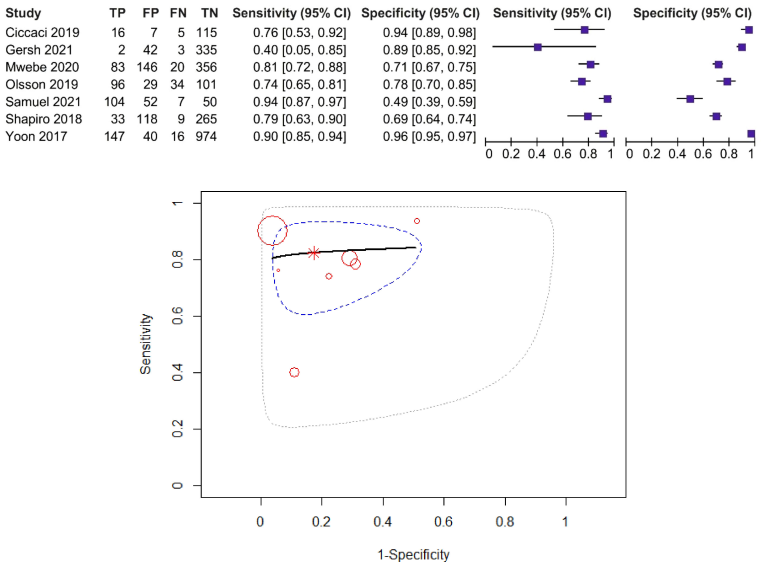

FIGURE 3
Forest plot and SROC curve (HIV patients, using the CRP threshold of 10 mg/L). The symbol * represents the pooled sensitivity and specificity.

our results demonstrate that CRP is an adequate screening test in regions with high prevalence of HIV infections. On the other hand, it is important to note the high quality for retrieved studies with a low risk of bias for the QUADAS-2 domains of participant sampling, index test, reference standard, and flow and timing for our research aim.

Although recent research indicates the use of immunological marker CRP as specific enough for distinguished TB diagnosis,

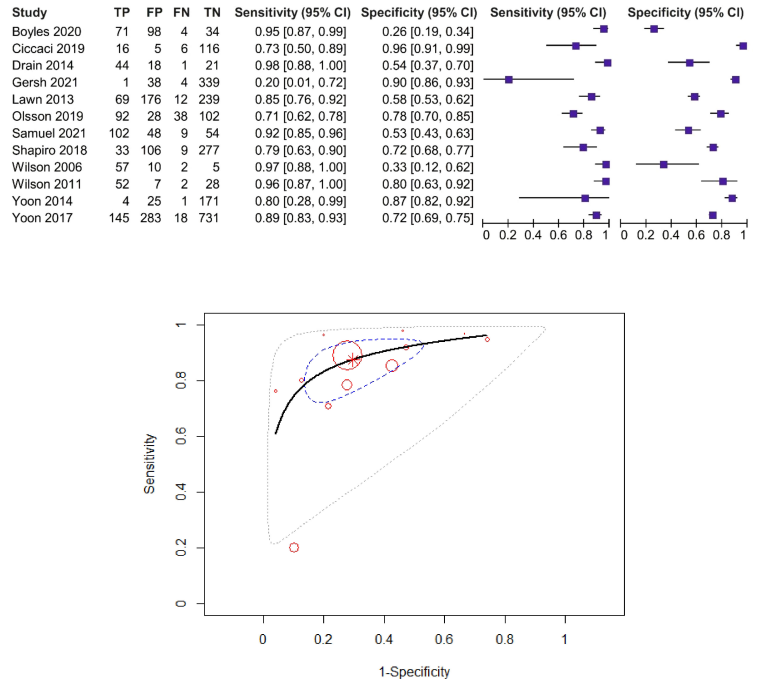

FIGURE 4
Forest plot and SROC curve (HIV patients, using the CRP threshold of 8 mg/L). The symbol * represents the pooled sensitivity and specificity.

this acute-phase protein can be especially relevant for monitoring the severity of the disease or the effectiveness of the treatment (41, 49, 50, 52). Along other host serum proteins such as alpha-2-macroglobulin, haptoglobin, fibrinogen, complement factor H, serum amyloid P, and transthyretin, CRP has recently been studied as a biosignature and point-of-care screening test for TB in HIV-infected patients from African settings (15). Fuster et al. analyzed the association between pro-inflammatory biomarkers (CRP, TNF-α, IL-6, IL-10, serum amyloid A, cystatin-C, and monocyte chemotactic protein-1) and mortality rates in HIV-diagnosed individuals and obtained positive statistical correlation (57). CRP can be instantly POC measured, saving time and without posing economic burden in comparison with TB symptom screening test or other molecular tests (41, 46, 49, 55). On the other hand, the faster PTB is diagnosed, the lower severity and mortality caused by this infection, especially in high-risk groups (such as HIV-infected patients) (16, 44, 46, 49, 58).

HIV-infected adults are disproportionately influenced by *M.tb.*, due to higher FN rates, lower sensitivity, and difficult accessibility in TB-endemic regions of screening tests (41, 42, 44, 47, 50). In high-burden areas, HIV infection deteriorates immune functions by lowering $CD4^+$ T cells while the increasing risk of primary *M.tb.* infection or reactivation in case of latent TB (22). HIV-infected individuals are 26 times more likely to be diagnosed with TB in comparison with those non-HIV infected (59). As *M.tb.* is characterized by an intriguing ability to adapt and survive on long term within the host even in case of immune responses and cytokine activation (60), HIV infection leads to deficiency in immune response ensuring a proper environment for TB development (22). Skogmar et al. found an inverse correlation between CRP and neopterin levels and CD4 cell count (61); in other words, increased inflammatory responses and immune activation are correlated with CD4 lymphocytopenia in adults infected with *M.tb* (60, 61). A superior inflammatory response and increased frequency of dissemination have been noticed in HIV-positive TB patients in comparison with non-TB patients; thus, even though CRP has shown insufficient values of sensitivity and specificity in screening for TB, rapid CRP test seems promising for exclusion of PTB in HIV-positive patients (41, 42, 46, 50). As shown, this could further facilitate differential diagnosis that could lead to rapid antiretroviral therapy and mortality reduction (46, 50). Moreover, Cicacci et al. confirm that higher bacillary load implies higher CRP levels and underlines a better specificity value of CRP than WHO symptom screening (16, 46, 49) or other cross-examined plasmatic inflammatory markers (16, 58). Other studies mentioned the importance of serum-analyzed CRP not only as a cost-effective method itself, but also as a potential test for reducing the employment of other molecular tests (16, 46, 49). Patients who had higher CRP values were more likely to be SSM-positive or detected by the GeneXpert MTB/RIF assay, which accentuated the prognostic utility of CRP as a potential screening PTB test (14, 16, 46, 53, 54).

The importance of establishing a threshold for CRP has also been mentioned, but some researchers emphasize that increasing the values of threshold (for example, from 5 to 10 mg/L) decreases, even more, the test sensitivity (with more than 10%) (47, 49, 54). The closest cut-point to WHO recommendations was obtained from the research conducted by Gersh et al.: CRP > 3.3 mg/L (sensitivity 80%, specificity 72%), while the cutoff point 10 mg/L led to decreased values for sensitivity (20%) concomitant with increased specificity (90%) (19). In other words, raising the analyzed CRP value could lead to a higher number of TB cases with improper prognostication (47), if other tests are not performed. CRP seems not to be conclusive as a singular TB diagnosis marker, but successful in facilitating systematic TB screening, when associated with the gold standard reference method or GeneXpert assay, within HIV-positive groups (49). This is the reason why the evaluation of CRP as a screening biomarker for active PTB has been widely analyzed and argued by researchers in the past years (11, 41, 47, 49, 52, 54), especially since the majority of the infected individuals, children, and adults are diagnosed with PTB (62–64).

WHO recommends the GeneXpert ® MTB/RIF assay as the initial diagnostic test in adults and children with presumed HIV-associated TB, rather than conventional microscopy and culture (1, 2). The LoopampTM MTBC Detection Kit is also recommended by WHO as a rapid diagnostic test to detect TB among people with signs or symptoms of TB (1). There is still no single rapid, accurate, and robust TB diagnostic test appropriate for use at the point of care (1), although diagnostics and reducing the time to introduce an adequate therapy are top priorities for WHO (56). Only the urine-based LF-LAM test was recommended in combination with existing TB tests to increase early TB diagnosis and treatment (1). WHO mentions sensitivity and specificity values for diagnostic methods as follows: 100% and 100% for liquid gold standard culture, 92% and 99% for Xpert Gene test, 61% and 98% for conventional sputum-smear microscopy, and 24% and 94% for chest radiography after negative sputum test or Xpert Gene test (8); our meta-analysis confirms 82% sensitivity and 82% specificity for CRP at 8 mg/L, underlining its accuracy as a screening strategy for active TB cases among HIV-positive patients.

Our research has several limitations. Most significantly, the reference test characteristics in this meta-analysis were not common across all studies, which is an important source of heterogeneity. Thus, there is no strong reference standard. The different reference standards among the article represent a source of potential bias. We have tried to separately evaluate different reference standards in order to predict the level of sensitivity and specificity compared to the gold standard

reference, but the included studies did not present sufficient data regarding the culture test. The different reference standards could have facilitated for a trade-off between yield of TB screen test and participants included in each analysis.

The outcome of this study could also have introduced bias due to the heterogeneous patient population or study design. For example, we evaluated the study conducted by Wilson et al. (2011) that included HIV-positive patients with less than 1 week of antitubercular therapy in comparison with the other studies that included only patients without previous antitubercular treatment. However, a culture conversion often appears after 1 month and up to 2 or 3 months of treatment in PTB active patients (9, 58, 64, 65), underlining that the consistent modifications in CRP values present low probability to appear after only 1 week of antitubercular therapy. Another limitation is that all studies were conducted in sub-Saharan Africa, and most studies were conducted in a single country (South Africa), particularly in settings with a high prevalence of HIV; thus, generalization of findings should be performed with care. This is also one of the reasons for heterogeneity in selected reference diagnostic standards of the included studies.

Although all the studies included in the meta-analysis were all conducted before the COVID-19 pandemic, it is important to mention the involvement of CRP in patients co-diagnosed with TB and COVID-19 with respiratory symptoms similar to those TB diagnosed (66). CRP values were significantly increased in individuals with severe infections, as Bostanghadiri and colleagues noted (66). Moreover, Parker et al. concluded that higher values of CRP were measured in patients diagnosed with all three infections: TB, HIV, and COVID-19 (67). However, researchers recommend CRP as a monitoring and prognosis tool in patients diagnosed with both TB and COVID-19, rather than as a rule-out screening test (68). Multiple investigations are required in order to generalize our results in those individuals.

Data availability statement

The raw data supporting the conclusions of this article will be made available by the authors, without undue reservation.

Author contributions

A-DM, AT-S, MB, and M-SS participated in research design and contributed to the data collection. AT-S undertook the statistical analysis and prepared the figures. RC, BSU, and BM interpreted the results from the analysis. A-DM and AT-S wrote the manuscript. BSU and C-GP reviewed the manuscript. All authors read and approved the final manuscript.

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Conflict of interest

The authors declare that the research was conducted in the absence of any commercial or financial relationships that could be construed as a potential conflict of interest.

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