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Major Article

Evaluation of the transcriptional immune biomarkers in peripheral blood from Warao indigenous associate with the infection by *Mycobacterium tuberculosis*

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Abstract

Introduction: Biomarkers are critical tools for finding new approaches for controlling the spread of tuberculosis (TB), including for predicting the development of TB therapeutics, vaccines, and diagnostic tools. **Methods:** Expression of immune biomarkers was analyzed in peripheral blood cells stimulated and non-stimulated with *M. tuberculosis* antigens ESAT-6, CFP10 and TB7.7. in Warao indigenous individuals. These biomarkers may be able to differentiate TB states, such as active tuberculosis (ATB) cases and latent tuberculosis infection (LTBI) from non-infected controls (NIC). A real-time reverse transcription polymerase chain reaction (RT-qPCR) assay was performed on 100 blood samples under non-stimulation or direct ex vivo conditions (NS=50) and stimulation conditions (S=50). **Results:** The findings are shown as the median and interquartile range (IQR) of relative gene expression levels of IFN-γ, CD14, MMP9, CCR5, CCL11, CXCL9/MIG, and uPAR/PLAUR immune biomarkers. MMP9 levels were significantly higher in the LTBI-NS and LTBI-S groups compared with the NIC-NS and NIC-S groups. However, CCR5 levels were significantly lower in the LTBI-S group compared with both NIC-NS and NIC-S groups. CCL11 levels were significantly lower in the LTBI-S group compared with the NIC-NS group. **Conclusions:** Preliminary findings showed that MMP9 immune biomarkers separated LTBI indigenous individuals from NIC indigenous individuals, while CCR5, CCL11, CD14, and IFN-γ did not differentiate TB states from NIC. MMP9 may be useful as a potential biomarker for LTBI and new infected case detection among Warao indigenous individuals at high risk of developing the disease. It may also be used to halt the epidemic, which will require further validation in larger studies.

Keywords: Tuberculosis. Warao indigenous. Immune biomarker. Real-time quantitative PCR (qPCR).

INTRODUCTION

Tuberculosis (TB) is one of the top 10 causes of death worldwide[1]. One third of the world's population is latently infected with *Mycobacterium tuberculosis* (Mtb), and up to 10% of infected individuals develop active TB in their lifetime[2-3]. Biomarkers are urgently needed to indicate progression from latent infection to clinical disease, to predict risk of reactivation after curing, and to provide accurate end points for drug and vaccine trials[4-6]. A recent study showed that 12 biomarkers were associated with the following clinical groups: "upstream" towards culture-positive TB on the TB disease spectrum (CD14, FCGR1A, FPR1, MMP9, RAB24, SEC14L1, and TIMP2), or "downstream" towards a decreased likelihood of TB disease (BLR1, CD3E, CD8A, IL7R, and TGFBR2). This suggested a correlation with *M. tuberculosis*-related pathology[7-8]. Matrix metalloproteinase or MMP activity is an essential component of resistance to pulmonary mycobacterial infection, and MMP9 specifically is required for macrophage

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recruitment and tissue remodeling to allow for the formation of tight, well-organized granulomas[8]. Other authors have identified the tissue inhibitor of metalloproteinases 1 (TIMP-1) as a diagnostic biomarker for TB diagnosis, with findings showing that plasma TIMP-1 and the relative mRNA expression levels may be a potential biomarker for TB diagnosis[9]. Recently, combinations of immune biomarkers such as CXCL9, sCD14, MMP9, and uPAR proteins were evaluated, in addition to anti-synthetic peptides ESAT-6 and Ag85A, covering certain sequences of *M. tuberculosis*. The findings showed that the anti-P-12034/uPAR combination could be a potential biomarker for identifying clinical TB patients with 96.7% sensitivity[10].

Tuberculosis is a public health problem among Warao communities of the Venezuelan delta[11-12]. We studied transcriptional immune biomarkers in Warao indigenous individuals by applying a real-time reverse transcription polymerase chain reaction (RT-qPCR) assay. We also studied the relative gene expression levels of: interferon-gamma (IFN-γ; the soluble phosphatidylinositol-linked membrane glycoprotein (sCD14); matrix metalloproteinases (MMP-9); CC receptor 5 (CCR5), which recognizes CXC chemokines such as MIP-1a, RANTES, and MIP-1b; CC chemokine eotaxin (CCL11), which signals through the CCR3 receptor, the monokine induced by IFN-γ (MIG/CXCL9); and the urokinase-type plasminogen activator receptor (uPAR/PLAUR). These were examined as tools for the diagnosis of active TB and for identifying the risk of developing active TB from latent infection.

METHODS

Subject recruitment and stratification

A transversal study was carried out among individuals of both sexes from the Warao indigenous population living in the Orinoco delta area. Indigenous groups were enrolled under strict clinical and laboratory criteria as described previously[13]. Subjects were stratified as follows: 1) Indigenous with active pulmonary tuberculosis (ATB, n=4), the latter according to clinical evaluation of smear and sputum culture-positive tuberculosis[14]; and 2) Healthy volunteer controls (n=46). Peripheral blood samples from all healthy indigenous controls without TB symptoms or disease activity were drawn for QuantiFERON–TB Gold In-Tube (QFT-IT) testing (Commercial test QuantiFERON–TB Gold In-Tube, Cellestis, Victoria, Australia). These indigenous individuals were additionally subjected to the tuberculin skin test (TST). The results were used as the basis for classification as the latent TB infection group (LTBI, n=18), which consisted of Warao indigenous individuals classified as QFT-IT positive (QFT-IT+, n=4) and TST positive (TST+, n=14). The healthy non-infected controls or Warao indigenous individuals were TST-negative (TST-) and QFT-IT negative (QFT-IT-), (NIC, n=28).

Ethical considerations

This study was approved by the Ethical Committee of the Biomedicine Institute. (Protocol number FONACIT-2013002319/2013-UNU-BIOLAC/2016). Voluntary informed consent forms were signed by all individuals.

Quantification of IFN-γ and tuberculin skin test

Measurement of IFN-γ levels in plasma was performed by an enzyme-linked immunosorbent assay (ELISA) using the QuantiFERON-TB Gold in tube or QFT-IT test kit, as previously reported[15].

A tuberculin skin test (TST) was carried out in Warao indigenous individuals without TB symptoms or disease activity. The TST was administered according to the Mantoux method, as previously reported[16].

Isolated Blood Cells and gene biomarker amplifications

Blood samples (n=100) under non-stimulation conditions (NS=50) and stimulation conditions (S=50) with *M. tuberculosis* antigens, Early Secretory Antigenic Target-6 (ESAT-6), Culture Filtrate Protein-10 (CFP10), and TB antigen 7.7 (TB7.7) were studied. Samples of 3–5 ml of peripheral blood were collected in a vacutainer tube with EDTA as an anticoagulant, and stimulated with antigens for 24 h at 37 °C. After incubation, both non-stimulated and stimulated blood cells were obtained by centrifugation. Total RNA was extracted from blood cells using the Total RNA Isolation System kit (Promega Corporation, WI, US) following the instructions of the supplier, and the RNA content was measured using a spectrophotometer at 260 nm. cDNA was generated from 5 µg of RNA using the Reverse Transcription System kit (Promega Corporation, WI US), following the manufacturer's protocol. The qPCR assay was performed as follows: 2 µl of cDNA (50 ng) was mixed with 8 µl of reaction cocktail mix, which was composed of 5 µl of SsoFast™ EvaGreen® 2x (BioRad, USA), 2.5 µl of sterile and nuclease-free water, and 0.25 µl of both forward and reverse primers of host immune biomarkers (20 µM each). Primers for selected genes including GAPDH were designed using the GenBank database and commercially synthesized by the Genomics Institute (IDT®, USA) for qPCR (**Table 1**). All primers were designed to anneal at 60 °C, and primer specificities and assay efficiencies were tested on control cDNA. The reaction mix was placed in a Light Cycler 480® (Roche Life Sciences, USA). Cycling parameters were 1 cycle for enzyme activation at 95 °C for 30 s, 45 cycles of amplification at 95 °C for 5 s and 60 °C for 10 s, 1 cycle for melting curve analysis at 60 °C and 90 °C for 15 s, and 1 cycle for cooling at 40 °C for 30 s.

Quantification of gene expression

The mRNA relative expression of each gene was calculated based on the values of Cp. Normalization of Cp was performed against corresponding values for the constitutive gene GAPDH, using the following equation: $2^{-\Delta\Delta ct}$ [17].

$$RE = 2^{-((CpProblem - CpGAPDH) - (CpProblem\ Control - CpGAPDH\ Control))}$$

Data analysis

Kruskal-Wallis and Dunn's multiple comparison tests were performed to determine differences among the ATB, LTBI, and NIC groups. Receiver operating characteristic (ROC)

TABLE 1: Primers for reverse transcription-quantitative polymerase chain reaction.

Gene	Direction	Sequence (5'-3')	Product Size (bp)	GeneBank accession no.
GAPDH	F	AGCCACATCGCTCAGACAC	66	NM-02046.3
	R	GCCCAATACGACCAAATCC		
IFN-γ	F	GTTTTGGGTTCTCTTGGCTGTTA	112	NM-00619.2
	R	AAAAGAGTTCCATTATCCGCTACATC		
CD14	F	GTTCGGAAGACTTATCGACCAT	95	NM-000591
	R	ACAAGGTTCTGGCGTGGT		
MMP9	F	GAACCAATCTCACCGACAGG	120	NM-004994
	R	GCCACCCGAGTGTAACCATA		
CCR5	F	GCCTCTGAATATGAACGGTGA	60	NM-00579.3
	R	ACATTTCCCTTCGTTGCTTC		
uPAR/PLAUR	F	CTGCAAGGGGAACAGCAC	118	NM-002659.3
	R	GCTTTGGTTTTTCGGTTCG		
MIG/CXCL9	F	GGAGTGCAAGGAACCCCAGTA	234	NM-002416
	R	CTT TTG GCT GAC CTG TTT CTC		
CCL11	F	CCCTTCAGCGACTAGAGAGC	90	NM-02986.2
	R	TATCCTTGGCCAGTTTGGTC		

Gene: **IFN-γ:** Interferon gamma cytokine; **sCD14:** the soluble phosphatidylinositol-linked membrane glycoprotein (sCD14); **MMP9:** Matrix metalloproteinases; **CCR5:** chemokine CC receptor 5; **CCL11:** CC chemokine Eotaxin; **F:** forward; **R:** reverse. Product size (bp) and Gene bank accession no.

curves and areas under the curves (AUC) were obtained to identify biomarkers with the best discriminatory accuracy. The significance level was set at 0.05. Statistical analyses were performed using GraphPad Prism, 6.0-Windows (San Diego, Ca. USA), SPSS (PASW Statistics ver. 18; SPSS, Chicago, IL, USA), and Epidat (Epidat ver. 3.1; Galicia, Spain) software.

RESULTS

Characteristics of study participants

Table 2 shows the demographic and clinical data for the participants. A total of 50 participants were enrolled into this study, 4 of whom were found to have alcohol/acid-fast bacilli and/or culture-positive pulmonary TB or active TB (ATB). Of 46 participants, 18 were classified as having latent TB infection (LTBI (QFT-IT+ or/and TST+), and 28 were healthy non-infected controls (NIC) with no reactivity to QFT-IT (QFT-IT-) or TST (TST-). The age results are shown as mean ± standard deviation (X - SD). The average age was 32.0–17.5 years for the ATB group, 34.0–15.6 years for the LTBI group, and 40.3–7.6 years for the NIC group (**Table 2**). There were no statistically significant differences between females and males, with differences of 1/3, 11/7, and 20/8 in the ATB, LTBI, and NIC groups, respectively (**Table 2**).

Bacteriological, QFT-IT, and TST

Results for the bacteriological, QFT-IT, and TST tests are shown in **Table 2**. Smears or smear plus culture were performed for all indigenous individuals with TB symptoms or disease activity (**Table 2**). Bacteriological assays revealed a statistically significant difference (P< 0.01) between the ATB and LTBI groups, both for smears as well as cultures (**Table 2**). QFT-IT results showed a statistically significant difference of P= 0.01 between indigenous QFT-IT+ and QFT-IT- in the LTBI group (4/18) and the NIC group (0/28) (**Table 2**). The skin reactivity test was carried out in all indigenous individuals without TB symptoms to study delayed-type hypersensitivity (DTH), for which a transversal diameter of indurations ³10mm were considered positive. There was a statistically significant difference of P< 0.01 between indigenous TST+ and TST- in the LTBI group (14/18) and the NIC group (0/28) (**Table 2**).

The mRNA expression levels of immune biomarkers by quantitative assay

The relative gene expression levels of immune biomarkers were detected by qPCR and normalized to GAPDH gene expression levels. Expression levels from the crossing point

TABLE 2: Characteristics of age, gender, bacteriological and immunological markers.

Markers	Patients (ATB) n=4	Contacts (LTBI) n=18	Controls (NIC) n=28	p-value
Mean ages in years	32.0±17.5	34.3±15.6	40.3±7.6	ns
Number of Female/Male	1/3	11/7	20/8	ns
Smear+ (%)	63.0[(a)]	0[(b)]	ND	0.0001
Culture+ (%)	100.0[(c)]	0[(d)]	ND	0.0001
QFT-IT + (%)	25.0	22.2[(e)]	0.0[(f)]	0.01
TST+ (%)	ND	88.8[(g)]	0.0[(h)]	0.0001

Indigenous individuals with active pulmonary TB (**ATB**). Indigenous individuals positive for QFT-IT/TST positive or with the latent TB infection (**LTBI**). Healthy non-infected controls (**NIC**). There were statistically significant differences between the percentage of smear ([(a)]&[(b)]), culture ([(c)]&[(d)]), the QFT-IT test ([(e)]&[(f)]), and the TST test ([(g)]&[(h)]). Not done (ND).

(Cp) of each gene were classified as very high (Cp < 25), high (Cp = 26-30), moderate (Cp = 31-35), low (Cp = 36-39), or not detected (Cp = 40). **Figure 1** shows comparisons of the medians and interquartile ranges (IQRs) of transcriptional immune biomarkers or the relative gene expression levels of MMP9, CCR5, CCL11, IFN-γ, and CD14 among the ATB, LTBI, and NIC groups. This was done for direct *ex vivo* or non-stimulated conditions (ATB-NS, LTBI-NS, and NIC-NS) and in *Mtb*-antigen stimulated conditions (ATB-S, LTBI-S, and NIC-S). With the use of Kruskal-Wallis and Dunn's multiple comparison tests for the medians of the transcriptional immune biomarker of MMP9, the results (mean - standard error (X - SE)) of the relative gene expression levels of MMP9 between the LTBI and NIC groups showed significantly higher MMP9 levels in the LTBI-NS group (150.7–74.4) than in the NIC-NS (21.74–15.20) and NIC-S (3.75–1.84) groups, with a significance of P< 0.01 and P< 0.01, respectively. There was also a significant difference in expression between the LTBI-S group (29.44–10.75) and the NIC groups, NIC-NS (21.74–15.20 (P= 0.02)) and NIC-S (3.75–1.84 (P< 0.01)) (**Figure 1A**).

The transcriptional immune biomarker in direct *ex vivo* and *Mtb*-antigen stimulated conditions of CCR5 levels are shown in **Figure 1B**. The Kruskal-Wallis and Dunn's multiple comparison tests for the medians of the transcriptional immune biomarker of the CCR5 relative gene expression levels showed significantly lower levels in the LTBI-S group (0.899–0.547) than in the NIC groups, NIC-NS (9.14–3.30, P< 0.01)) and NIC-S (7.20–2.04, P< 0.01)) (**Figure 1B**). A Spearman's rank correlation analysis determined a negative association between CCR5 and MMP9 when LTBI-NS and LTBI-S groups were compared (rho = -0.5743, P= 0.03 and rho = -0.5756, P= 0.03, respectively).

The relative gene expression levels of CCL11 and the transcriptional immune biomarker in direct *ex vivo* and *Mtb*-antigen stimulated conditions are shown in **Figure 1C.** The Kruskal-Wallis and Dunn's multiple comparison tests for the medians of the transcriptional immune biomarker of the relative CCL11 gene expression levels showed significantly lower CCL11 levels in the LTBI-S group (1.68 - 1.26) than in the NIC-NS group (7.33 - 2.39 (P< 0.01)) (**Figure 1C**).

The Kruskal-Wallis and Dunn's multiple comparison tests for the medians of the transcriptional immune biomarkers of IFN-γ and sCD14 did not show statistically significant differences among the groups (**Figure 1D and 1E)**. In addition, there was complete absence of expression for the selected gene for qPCR to determine the gene expression levels of MIG/CXCL9 and uPAR/PLAUR immune biomarkers. This is probably due to the primer sequences used, and therefore further studies must be performed to establish whether different primers determine the mRNA expression levels of these immune biomarkers.

Receiver operating characteristic analysis of selected genes

Receiver operating characteristic (ROC) curves were constructed for evaluating immune biomarker diagnostic powers to distinguish LTBI indigenous individuals from NIC indigenous individuals. **Figure 2** shows ROC curve comparison analyses. The area under curve - standard error (AUC - SE) for each immune biomarker was: MMP9 (AUC= 0.771–0.080: 0.613–0.928 95% CI, P< 0.01), CCR5 (AUC= 0.317–0.090: 0.139–0.494 95% CI, P= 0.06), CCL11 (AUC= 0.352–0.101: 0.154–0.550 95% CI, P= 0.13), IFN-γ (AUC= 0.429–0.100: 0.233–0.625 95% CI, P= 0.47) and sCD14 (AUC= 0.609–0.106: 0.402–0.817 95% CI, P= 0.27) (**Figure 2**).

Sensitivity and specificity tests

Table 3 illustrates the sensitivity and specificity of the immune biomarkers for diagnosis of *Mtb* infection. Statistical analyses demonstrated that sensitivities ranged between 94.12% and 21.43%, while specificities ranged between 88.46% and 23.08%. The MMP9 immune biomarker provided the best sensitivity (94.12%) for diagnosing LTBI but had 37.04% specificity for detecting subjects without *Mtb* infection. The positive predictive value was 48.48 (29.92/67.05), and the negative predictive value was 90.91 (69.37/100.0), (**Table 3**). The CCL11 immune biomarker provided the sensitivity of 46.15% and specificity of 23.08%. The positive predictive value was 23.08 (4.96/41.19) and the negative predictive value was

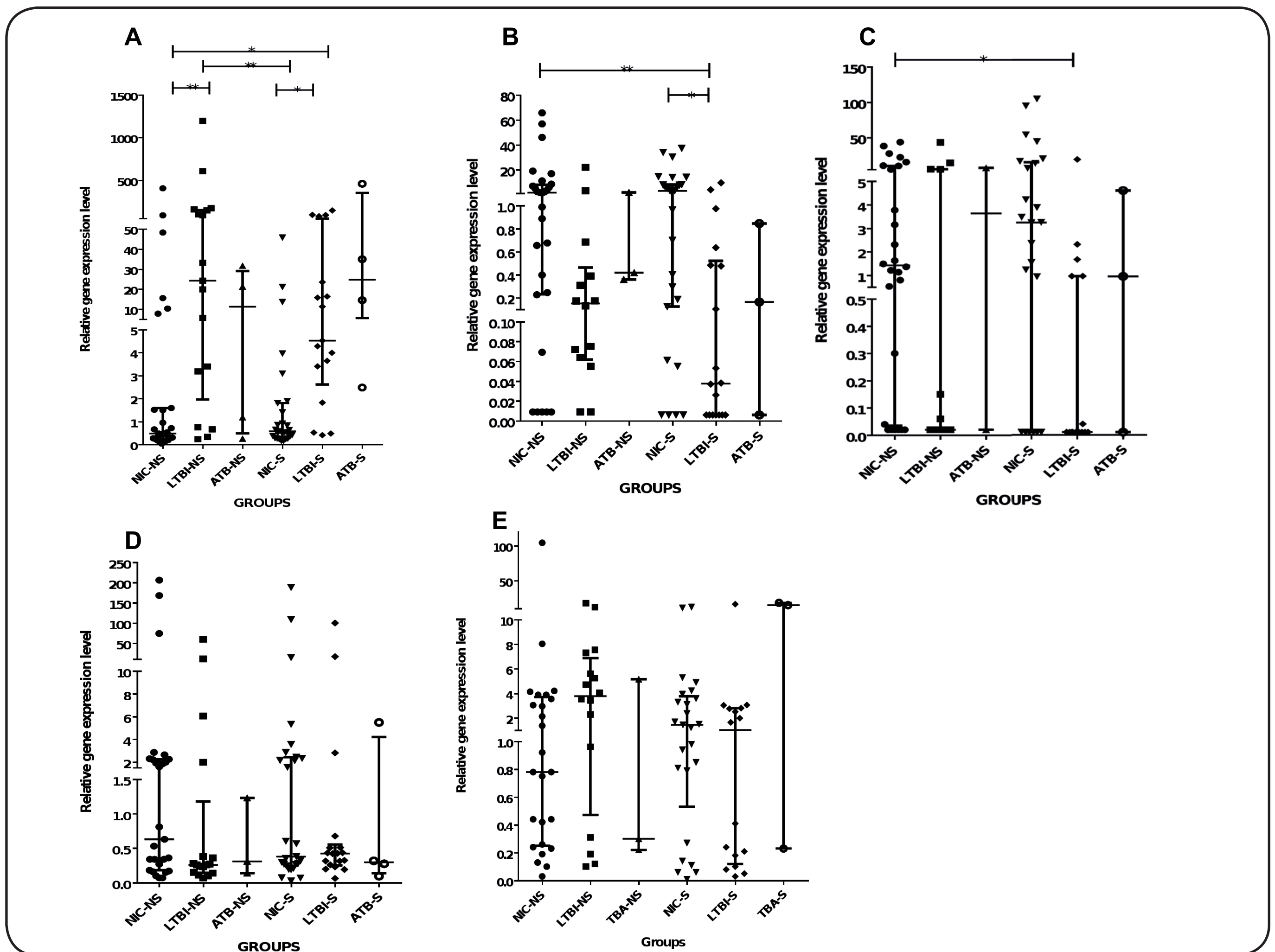


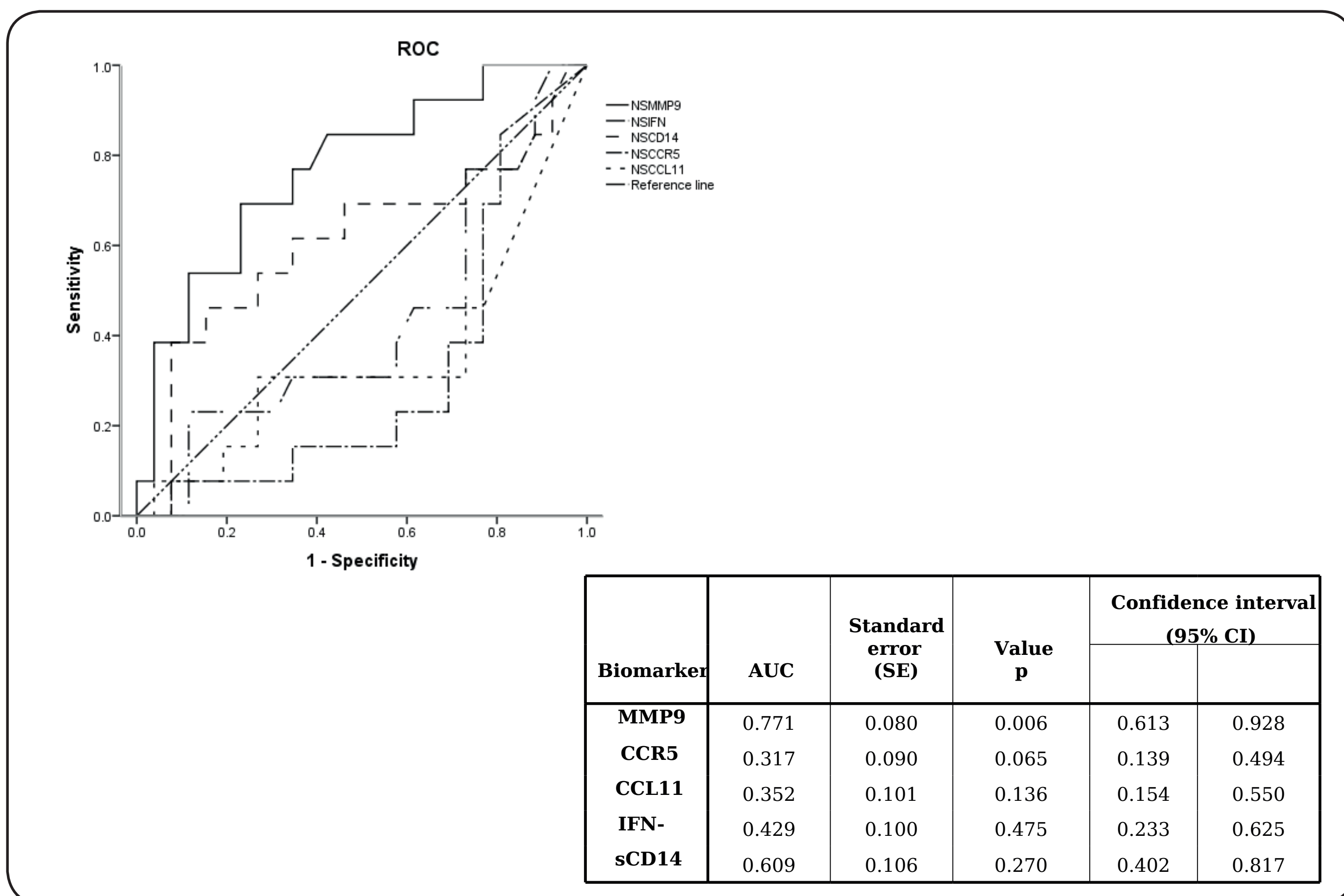
FIGURE 1: Relative expression of immune biomarkers between groups studied. Horizontal bars are medians and interquartile range (IQR) of IFN-γ for non stimulated (NS) conditions; NIC-NS (●), LTBI-NS (■) and ATB-NS (▲) and for stimulated conditions (S); NIC-S (▼), LTBI-S (◆) and ATB-S (○). Kruskal-Wallis and Dunn's multiple comparison tests were used to compare the groups. (2A)*** Represents significance of differences for the MMP9 levels in the LTBI-NS and LTBI-S groups compared with the NIC-NS groups, P= 0.0006 and P= 0.0009, respectively, and NIC-S groups, P= 0.0002. (2B)*** and ** Represent significance of differences for the CCR5 levels in the LTBI-S group compared with the NIC-NS group, P= 0.0006, and in the LTBI-S group compared with the NIC-S group, P= 0.002. (2C)** Represents significance differences for the CCL11 levels in the LTBI-S group compared with the NIC-NS group, P= 0.001.

46.15 (15.21/77.1), (**Table 3**). The sCD14 immune biomarker provided the sensitivity of 43.75% and specificity of 88.46%. The positive predictive value was 70.0 (36.6/100.0) and the negative predictive value was 71.88 (54.73/89.02), (**Table 3**). The IFN-γ immune biomarker provided the sensitivity of 35.29% and specificity of 29.63%. The positive predictive value was 24.0 (5.26/42.74) and the negative predictive value was 42.11 (17.27/66.94), (**Table 3**). The CCR5 immune biomarker provided the sensitivity of 21.43% and specificity of 28.57%. The positive predictive value was 13.04 (0.0/28.98 95% CI) and the negative predictive value was 42.11 (17.27/66.94 95% CI), (**Table 3**).

DISCUSSION

Several transcriptional immune biomarkers detected in direct *ex vivo* conditions have been evaluated in adult individuals with a diagnosis of TB. In addition, LTBI or PPD+ and non-infected individuals or PPD- have been detected using a Custom Array 4X2K "UIMZ-IMSS-MX microarray[18-19]. Authors identified 108 genes in Mexican ATB subjects. Serum proteins produced by these genes were over-expressed in whole blood from ATB patients. This made them excellent candidates for biomarkers, as the gene elevations could differentiate ATB patients from LTBI or NIC individuals without infection[19].

Immunogenicity factors that contribute to susceptibility and progression of pulmonary TB among Warao indigenous individuals provided an opportunity to study these individuals, as different genetic backgrounds exist between Warao indigenous individuals and white Americans[20-21]. In the present study, differential gene expression in indigenous individuals with *Mtb* infection versus healthy indigenous individuals was performed. The results showed that for the medians of the relative MMP9 gene expression levels, there was no statistically significant difference between the ATB and NIC groups in direct *ex vivo*



Biomarker	AUC	Standard error (SE)	Value p	Confidence interval (95% CI)	
MMP9	0.771	0.080	0.006	0.613	0.928
CCR5	0.317	0.090	0.065	0.139	0.494
CCL11	0.352	0.101	0.136	0.154	0.550
IFN-	0.429	0.100	0.475	0.233	0.625
sCD14	0.609	0.106	0.270	0.402	0.817

FIGURE 2: Receiver operating characteristic curve (ROC) analyses of immune biomarker tests. ROC analyses were constructed for evaluating immune biomarker diagnostic powers to distinguish between LTBI and NIC indigenous individuals. The area under the curves (AUC), standard error (SE), p value and 95% confidence interval (95% CI) of immune biomarkers; IFN-γ, sCD14, MMP9, CCR5 and CCL11.

or non-stimulated and stimulated conditions. However, between the LTBI and NIC groups, there were statistically significant differences between the high MMP9 levels for the LTBI-NS and LTBI-S groups compared with the low MMP9 levels for the NIC-NS and NIC-S groups. The high MMP9 levels associated with TST+ in LTBI-NS and LTBI-S groups, or indigenous individuals, compared with the low MMP9 levels for the NIC-NS and NIC-S groups, or healthy indigenous individuals, was notable. This could correlate with findings that support the idea that early MMP activity is an essential component of resistance to pulmonary mycobacterial infection, and that MMP9 specifically is required for macrophage recruitment and tissue remodeling for the formation of tight, well-organized granulomas[22]. Authors reported that following aerosol infection with *Mtb*, dissemination of bacilli occurred earlier in the C57BL/6 resistant mouse strain than in the susceptible CBA/J strain, as was evident from an increased number of bacteria in the blood, spleen, and liver at day 14 after infection. Non-specific blocking of MMPs in C57BL/6 mice early during infection reduced hematogenous spread of the bacilli, suggesting that MMPs indeed play a role in facilitating dissemination[23].

A recent *ex vivo* transcriptional immune biomarker study was performed in a cohort of 99 Indian children with intrathoracic tuberculosis and during anti-TB treatment. Expression of three genes down-regulated during anti-TB treatment (FCGR1A, FPR1, and MMP9) exhibited a positive correlation with the extent of TB disease, whereas expression of eight up-regulated genes (BCL, BLR1, CASP8, CD3E, CD4, CD19, IL-7R, and TGFBR2) exhibited a negative correlation with the extent of disease[23]. A study restricted to Ethiopian adult TB patients and household contacts also identified MMP9 in addition to BLR1, FCGR1A, and IL7R as differentiating markers[24]. This agreement across different populations strengthens the likelihood of the MMP9 immune biomarker being relevant to a future diagnostic test for TST+ in Warao indigenous individuals. Taken together; the transcripts of MMP9 provided the highest sensitivity of 94.12% for diagnosing LTBI.

In regards to the CCR5 immune biomarker, comparison tests for the medians of CCR5 showed that between the LTBI and NIC groups, there were statistically significant differences between the low CCR5 levels in the LTBI-S group compared with the high CCR5 levels in the NIC-NS and NIC-S groups. In addition, there were no statistically significant differences between the NIC and ATB groups for comparison tests for the medians of the CCR5 transcriptional immune biomarker. This was probably due to the low number of ATB indigenous

TABLE 3: Diagnostic accuracy of the immune biomarker tests.

	MMP9	CCL11	sCD14	IFN-γ	CCR5
Sensitivity (%)	94.12 (79.99/100.0)	46.15 (15.21/77.1)	43.75 (16.32/71.18)	35.29 (9.64/60.95)	21.43 (0.0/46.49)
Specificity (%)	37.04 (16.97/57.1)	23.08 (4.96/41.19)	88.46 (74.26/100)	29.63 (10.55/49.71)	28.57 (10.05/47.09)
PPV (%)	48.48 (29.92/67.05	23.08 (4-96/41.19)	70.0 (36.6/100)	24.0 (5.26/42.74)	13.04 (0.0/28.98)
NPV (%)	90.91 (69.37/100.0)	46.15 (15.21/77.1)	71.88 (54.73/89.02)	42.11 (17.27/66.94)	42.11 (17.27/66.94)
LR +	1.49 (1.09/2.04)	0.6 (0.32/1.12)	3.79 (1.14/12.6)	0.5 (0.25/1.0)	0.3 (0.11/0.84)
LR –	0.16 (0.02/1.13)	2.33 (0.98/5.53)	0.64 (0.4/1.0)	2.18 (1.11/4.31)	2.75 (1.44/5.25)
YI	0.31 (0.1/0.53)	-0.312 (-0.62/-0.01)	0.32 (0.05/59)	-0.35 (-0.64/-0.07)	-0.5 (-0.77/-0.23)
COV	0.315	5.355	4.165	0.310	0.393

Sensitivity, Specificity, **PPV:** Positive predictive value; **NPV:** Negative predictive value; **LR+:** Likelihood ratio (positive); **LR-:** Likelihood ratio (negative); **YI:** Youden index; **COV:** Cut off value.

individuals included in the study. During *Mtb* infection, the pathogen modulates C-C Chemokine Receptor 5 (CCR5) to enhance IL-10 production, indicating the possible involvement of CCR5 in regulation of the host immune response. These events culminate in up-regulation of the production of immunosuppressive cytokine IL-10, which is associated with the down-regulation of macrophage MHC-II expression and up-regulation of CCR5 expression via engagement of STAT-3 in a positive feedback loop[25]. The CCR5 immune biomarker has been reported as an immunological marker that differentiates between different outcomes of *Mtb* infection. CCR5, as well as CXCR4, has been connected with the diagnosis of the extent of TB disease[25-27]. When we used Spearman's rank correlation analysis, a significant negative association between MMP9 and CCR5 in the LTBI-NS and LTBI-S groups was determined (rho = -0.5743 and rho = -0.5756, respectively). This indicates that when MMP9 increases, CCR5 decreases, suggesting that CCR5 is not useful for detecting infected cases among adult Warao indigenous individuals at high risk of developing the disease. Considering that low CCR5 levels were found in the LTBI-S group compared with the high CCR5 levels in the NIC-NS and NIC-S groups, further studies must be performed to rule out the possibility that polymorphisms in this gene may influence the low levels of CCR5 found among the LTBI groups. Further studies are also necessary to determine whether expression of this gene could be diminished by the influence of *Mtb* infection.

In regards to the results for the gene expression levels of CCL11, these showed a statistically significant difference between the low CCL11 levels in the LTBI-S group compared with the high CCL11 levels in the NIC-NS group. In addition, there was no statistically significant difference between the ATB and NIC groups. Eotaxin or CCL11 is a CC-chemokine that signals through the CCR3 receptor. It is produced by IFN-γ -stimulated endothelial cells and TNF-activated monocytes[28]. It has been reported that CCL11, CCL24, and CCL26, which are produced by Th2 cells and other cells that induce Th2 development, are increased in TB patients compared to the controls. It appears that TB suppresses Th1 and subsequently, the classic function of macrophages, by inducing chemokine expression[28]. Studies must be conducted to understand the statistically significant differences found for CCL11 levels among the indigenous individuals that comprised the LTBI group.

The IFN-γ immune biomarker was expressed at low levels in all groups. Thus, comparisons of the medians and the relative gene expression levels of IFN-γ did not show statistically significant differences among the groups, neither in direct *ex vivo* or non-stimulated and stimulated conditions. The present IFN-γ findings correlate with those of our previous study, which examined the capacity of antigen-induced proliferation by PBMCs and IFN-γ production in Warao indigenous individuals with pulmonary TB and in healthy controls. We previously reported on the Creole population in another larger cohort of Venezuelan adults. In the previous study, IFN-γ production in Warao patients and controls was significantly lower after stimulation for 24 h and 48 h compared with that in the Creole group[13]. Studies in mice and humans showed that IFN-γ, Il-12, and TNF-α are cytokines involved in the control of *Mtb* infections[29,30]. Several studies have evaluated the diagnostic potential of cytokine biomarkers for discrimination between TB infection states. Authors have identified cytokines as host biosignatures (IL-17F, MIP-3a, IL-13, IL-17A, IL-5, IL-9, IL-1, IL-2, and IFN-γ) that could identify and uniquely discriminate between TB infection states[31,32].

For the median results of the relative sCD14 gene expression levels for discrimination between TB infection states and controls, there were no statistically significant differences among the studied groups. Though the comparison tests for the medians of the transcriptional immune biomarkers of sCD14 showed high levels of this immune biomarker in the ATB-S group compared with the ATB-NS and the LTBI-S and NIC-S groups, this was probably due to the low number of ATB indigenous individuals included in the study.

A recent study focused on delineating the immune factors associated with the asymptomatic states comprising latent tuberculosis infection (LTBI)[33]. To broadly characterize the immune state, latently infected and uninfected control individuals from South Africa were analyzed by CyTOF and flow cytometry. The results showed no significant changes in peripheral monocyte or lymphocyte counts compared to uninfected controls. Significant differences were identified in the percentage of immune effector cell subsets between samples from uninfected controls and individuals with LTBI. Granzyme B (GZMB) and perforin (PRF)-expressing cells were significantly higher in individuals with LTBI. These mostly consisted of NK cells and GZMB+PRF+IFNγ+TNF+ polyfunctional cells, which largely comprised CD27−CD8+ αβ T cells, but also included NK cells and γδ T cells. Authors therefore showed that latent tuberculosis is associated with enhanced cytotoxic responses, which are mostly mediated by CD16 (also known as FcγRIIIa) and natural killer cells, and continuous inflammation coupled with immune deviations in both T and B cell compartments[33]. Furthermore, authors using cell-type deconvolution of transcriptomic data from several cohorts of different ages, genetic backgrounds, geographical locations, and infection stages showed that although deviations in peripheral B and T cell compartments generally start at latency, they are heterogeneous across cohorts. However, an increase in the abundance of circulating natural killer cells in tuberculosis latency, with a corresponding decrease during active disease and a return to baseline levels upon clinical cure, are features that are common to all cohorts. Furthermore, by analysing three longitudinal cohorts, it was found that changes in peripheral levels of natural killer cells can inform disease progression and treatment responses, and inversely correlate with the inflammatory state of the lungs of patients with active tuberculosis. Together, these findings offer crucial insights into the underlying pathophysiology of tuberculosis latency, and identify factors that may influence infection outcomes[33].

No studies applying gene expression profiling of Warao indigenous adults with TB infection states have been published so far. Authors have reported a study which identified a 116-gene signature set for discriminating TB and LTBI among Warao indigenous children[34]. Ten genes selected that are involved in calcium signaling and calcium metabolism were highly discriminative between TB, LTBI, and healthy controls. After validation by qPCR, 5 of the 10 genes (S100P, HBD, PIGC, CHRM2, and ACOT7) were sufficient to classify with 78% sensitivity and 96% specificity of TB cases correctly with no LTBI indigenous children wrongly classified as having TB[34].

Regarding TB diagnostics, progress towards elimination of tuberculosis has remained elusive despite intensified standard measures of control. After a period of global acceleration in 2001–05, the case detection rate worldwide decelerated in 2006 and 2007, reaching 63% in 2007. Thus, the target of a case detection rate of at least 70% by 2005 has not yet been achieved, and is unlikely to be met until 2014. Insufficient access to advanced diagnostic tests (real-time reverse transcription polymerase chain reaction (RT-qPCR), flow cytometry, lateral-flow assay, CyTOF, etc.) has contributed to this suboptimal performance. Furthermore, national tuberculosis programs in disease endemic countries continue to rely largely on antiquated and inaccurate methods, such as direct smear microscopy, solid culture, chest radiography, and tuberculin skin testing. There is no rapid, point-of-care test that allows early detection of active tuberculosis at health clinics. Diagnostic delays, misdiagnosis, and inadequate implementation of existing tests result in increased morbidity and mortality in patients, and allow continued transmission of TB.

Insufficient access to advanced diagnostic tests in countries with limited resources such as Venezuela is an ongoing issue. However, we consider the next approach is to provide access to advanced diagnostic tests such as CyTOF. This is a proteomics technology that accesses the abundance of cell subsets, protein expression, and activation of signaling pathways at the single-cell level, as reported by Roy Chowdhury R et al.,[33]. Application of qRT-PCR detection of immune biomarkers at the mRNA level allowed us to establish the diagnostic potential of 7 immune biomarkers studied. Of these, MMP9 could identify LTBI indigenous from healthy indigenous with 94.12% sensitivity. Therefore, MMP9 has potential for further development and studies for confirming the diagnostic accuracy of the proposed biomarker. We consider that the analysis of MIG/CXCL9, uPAR/PLAUR, and alternative biomarkers consistently associated with the different stages of *M. tuberculosis* infection could be done using CyTOF technology. This could offer

a better understanding of the immune state of latent *M. tuberculosis* infection to active disease, in addition to understanding the relevance of these biomarkers in a future point-of-care (POC) or a diagnostic POC test. If validated, this would contribute significantly to indicating progression from latent infection to clinical disease, in addition to predicting risk of reactivation after cure, especially in Warao indigenous TB-endemic communities.

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Conflict of Interest: The authors declare that there is no conflict of interest.

REFERENCES

1. World Health Organization (WHO). Global tuberculosis report 2017. Geneva: WHO; 2017. Available from: http://www.who.int/tb/publications/global_report/gtbr2017_main_text.
2. Foulds J, O'Brien R. New tools for the diagnosis of tuberculosis: the perspective of developing countries. Int J Tuber Lung Dis. 1998;2(10):778-83.
3. Perkins MD. New diagnostic tools for tuberculosis. Int J Tuberc Lung Dis. 2000;4(suppl 2); 182-88.
4. Walzl G, McNerney R, du Plessis N, Bates M, McHugh TD, Chegou, et al. Tuberculosis: advances and challenges in development of new diagnostics and biomarkers. Lancet Infect Dis. 2018;18(7):e199-e210.
5. Wallis RS, Pai M, Menzies D, Doherty TM, Walzl G, Perkins MD, et al. Biomarkers and diagnostics for tuberculosis: progress, needs, and translation into practice. Lancet. 2010;375(9729):1920-37.
6. Abramo C, Meijgaarden KE, Garcia D, Franken KLMC, Klein MR, Kolk AJ, et al. Monokine induced by interferon gamma and IFN-γ response to a fusion protein of *Mycobacterium tuberculosis* ESAT-6 and CFP-10 in Brazilian tuberculosis patients. Microbes Infect. 2006;8(1):45-51.
7. La Manna MP, Orlando V, Li Donni P, Sireci G, Di Carlo P, Cascio A, Dieli F, et al. Identification of plasma biomarkers for discrimination between tuberculosis infection/disease and pulmonary non tuberculosis disease. PLoS One. 2018;13(3):e0192664.
8. Jenum S, Dhanasekaran S, Lodha R, Mukherjee A, Kumar Saini D, Singh S, et al. Approaching a diagnostic point-of-care test for pediatric tuberculosis through evaluation of immune biomarkers across the clinical disease spectrum. Sci Rep. 2016;6:18520-30.
9. Chen Y, Wang J, Ge P, Cao D, Miao B, Robertson I, et al. Tissue inhibitor of metalloproteinases 1, a novel biomarker of tuberculosis. Mol Med Rep. 2017;15(1):483-7.
10. Araujo Z, Macias-Segura N, López-Ramos JE, de Waard JH, Vanegas M, Patarroyo MA, et al. Diagnostic accuracy of combinations of serological host biomarkers for identifying clinical tuberculosis. J Infec Dev Ctries. 2018;12(6):429-41.
11. Ministerio de Sanidad y Asistencia Social (MSAS). Programa Integrado de Control de la Tuberculosis. Seminario Técnico-Administrativo. Caracas: 1999. 150 p.
12. Ministerio de Salud y Desarrollo Social (MSDS). Evaluación del Programa Nacional de Control de la Tuberculosis. Año Evaluado 2006. Caracas: 2006. 200 p.
13. Giampietro F, de Waard JH, Rivas-Santiago B, Enciso-Moreno JA, Salgado A, Araujo Z. *In vitro* levels of cytokines in response to purified protein derivative (PPD) antigen in a population with high prevalence of pulmonary tuberculosis. Hum Immunol. 2010; 71(11):1099-104.
14. Kudoh S, Kudoh T. A simple technique for culturing tubercle bacilli. Geneva Bull World Health Organ. 1974;51(1):71-82.
15. Enciso-Moreno JA., Monarrez-Espino J. Performance of Tuberculin Skin Test Compared to QFT-IT to Detect LatentTB Among High-risk Contacts in Mexico. Archives of Medical Research 2013; 44: 242-248.
16. Arnadottir T, Rieder HI, Trebuq A, Waaler H. Guidelines for conducting tuberculin skin test surveys in high prevalence countries. Tuber Lung Dis. 1996;77(suppl 1):1-19.
17. Livak KJ, Schmittgen TD. Analysis of relative gene expression data using real time quantitative PCR and the 22DDCT method. Methods. 2001;25(4):402-8.
18. Walzl G, Ronacher K, Hanekom W, Scriba TJ, Zumla A. Immunological biomarkers of tuberculosis. Nat Rev Immunol. 2011;11(5):343-54.
19. Lopez-Ramos JE, Macias-Segura N, Cuevas-Cordoba B, Araujo-Garcia Z, Bastian Y, Castañeda-Delgado JE, et al. Improvement in the diagnosis of tuberculosis combining *Mycobacterium tuberculosis* immunodominant peptides and serum host biomarkers. Arch Medic Res. 2018;49(3):147-53.
20. Layrisse Z, Heinen HD, Balbas O, García E, Stoikow Z. Unique HLA-DR/DQ associations revealed by family studies in Warao Amerindians. Haplotype and homozygosity frequencies. Hum Immunol. 1988;23(1):45-57.
21. Makhatadze NJ, Franco MT, Layrisse Z. HLA class I and class II allele and haplotype distribution in the Venezuelan population. Hum Immunol. 1997;55(1):53-8.
22. Elkington PT, Ugarte-Gil CA, Friedland JS. Matrix metalloproteinases in tuberculosis. Eur Respir J. 2011;38:456-64.
23. Taylor JL, Hattle JM, Dreitz SA, Troudt JM, Izzo LS, Basaraba RJ, et al. Role for matrix metalloproteinase 9 in granuloma formation during pulmonary *Mycobacterium tuberculosis* infection. Infect Immun. 2006;74(11):6135-44.
24. Jenum S, Bakken R, Dhanasekaran S, Mukherjee A, Lodha R, Singh S, et al. BLR1 and FCGR1A transcripts in peripheral blood associate with the extent of intrathoracic tuberculosis in children and predict treatment outcome. Sci Rep. 2016;6:38841-51.
25. Das S, Banerjee S, Majumdar S. Immune subversion by *Mycobacterium tuberculosis* through CCR5 mediated signaling: Involvement of IL-10. PLoS One. 2014;9(4):e92477.
26. Wolday D. Expression of chemokine receptors CCR5 and CXCR4 on CD4+ T cells and plasma chemokine levels during treatment of active tuberculosis in HIV-1-coinfected patients. J Acquir Immune Defic Syndr. 2005;39(3):265-71.
27. Carpenter D, Taype C, Goulding J, Levin M, Eley B, Anderson S, et al. CCL3L1 copy number, CCR5 genotype and susceptibility to tuberculosis. BMC Med Genet. 2014;15:5-13.
28. Sharifabadi RA, Hassanshahi G, Ghalebi SR, Arababadi MK, Khorramdelazad H, Zainodini N, et al. All eotaxins CCL11, CCL24 and CCL26 are increased but to various extents in pulmonary tuberculosis patients. Clin Lab. 2014;60(1):93-7.
29. Mihret A, Markos Abebe M. Cytokines and Chemokines as Biomarkers of Tuberculosis. J Mycobac Dis. 2013;3(2):1000128.
30. Flynn JL, Chan J, Triebold KJ, Dalton DK, Stewart TA, Bloom BR. An essential role for interferon gamma in resistance to *Mycobacterium tuberculosis* infection. J Exp Med. 1993; 178(6):2249-54.
31. Chegou NN, Heyckendorf J, Walzl G, Lange Ch, Ruhwald M. Beyond the IFN-γ horizon: biomarkers for immunodiagnosis of infection with *Mycobacterium tuberculosis*. Eur Respir J. 2014;43(5):1472-86.

32. Kamakia R, Kiazyk S, Waruk J, Meyers A, Ochanda J, Ball TB, et al. Potential biomarkers associated with discrimination between latent and active pulmonary tuberculosis. Int J Tuberc Lung Dis. 2017;21(3):278-85.
33. Roy Chowdhury R, Vallania F, Yang Q, Lopez Angel CJ, Darboe F, Penn-Nicholson A, et al. A multi-cohort study of the immune factors associated with M. tuberculosis infection outcomes. Nature. 2018;560(7720):644-8.
34. Verhagen LM, Zomer A, Maes M, Villalba JA, del Nogal B, Eleveld M, et al. A predictive signature gene set for discriminating active from latent tuberculosis in Warao Amerindian children. BMC Genomics. 2013;14:74-85.

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