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Original Article

Diagnostic potential of combining plasma biomarkers of tissue damage and inflammation in pediatric TB

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Abstract *Introduction:* Immune-based diagnostic tests for tuberculosis (TB) have suboptimal sensitivity in children and cannot differentiate between latent infection (LTBI) and active disease. This study evaluated the diagnostic potential of a broad range of biomarkers of tissue damage and inflammation in unstimulated plasma in children.
Methods: We analyzed 17 biomarkers in 15 non—*M. tuberculosis* (MTB)-infected controls and 33 children with TB infection (LTBI, n = 8; probable TB, n = 19; confirmed TB, n = 6). Biomarker concentrations were measured using a Luminex magnetic bead—based platform and multiplex sandwich immunoassays. Concentrations, correlations and diagnostic accuracy assessments were conducted among patient groups.
Results: Confirmed TB cases had significantly higher concentrations of IFN-γ and IL-2 and higher IFN-γ/MCP-1 and IL-2/MCP-1 ratios compared to LTBI and non—MTB-infected children. Among children with confirmed TB, there was a strong correlation between IFN-γ and IL-10 ($r = 0.95$; $p < 0.001$) and a significant correlation between IL-2 and IL-1ra ($r = 0.92$), IL-21 ($r = 0.91$), MCP-3 ($r = 0.84$), and MMP-1 ($r = 0.85$). The IFN-γ/MCP-1 ratio was the most accurate biomarker combination for differentiating between MTB-infected and non—MTB-infected children (AUC, 0.82; sensitivity, 87.9%; specificity, 66.6%; $p < 0.001$) and between active TB and non—MTB-infected children (AUC 0.82; sensitivity 88.0%; specificity 60.0%; $p < 0.001$). None of the biomarkers investigated were able to discriminate between LTBI and active TB.
Conclusion: Our data suggest that combining the analyses of multiple biomarkers in plasma has the potential to enhance diagnosis of TB in children and, thus, warrants additional investigation. In particular, the diagnostic potential of IFN-γ/MCP-1 ratios should be further explored in larger pediatric cohorts.

Introduction

Tuberculosis (TB) remains one of the most prevalent infectious diseases worldwide. In 2022, there were 1.3 million deaths and 10.6 million cases, including 1.1 million cases in children under the age of 15 years, equivalent to 16% of the total.[1] In recent years, the COVID-19 pandemic resulted in an increase in the incidence and mortality of TB due to limited access to healthcare and diagnostic delays, and it will likely take years to return to previous rates.[2] This dire situation especially affects children, in whom the absence of reliable diagnostic tests poses a particular challenge.[3]

Current TB tests implemented in clinical practice are less sensitive in children than in adults and require complex infrastructure, which precludes their use at the point of care.[4] Interferon-gamma (IFN-γ) release assays (IGRAs) are subject to considerable limitations in children, including low sensitivity (approximately 60—80%) in active TB[5,6] and increased rates of indeterminate results.[7] In addition, IGRAs cannot be performed on native blood samples, but require a stimulation step, which in clinical practice typically leads to results not being available for 2—3 days, even in high-throughput laboratories. Furthermore, IGRAs are unable to differentiate between latent TB infection (LTBI) and active disease.[8] Molecular assays, such as the Xpert MTB/RIF Ultra assay (Cepheid, Sunnyvale, CA, U.S.A.), enable rapid detection of *M. tuberculosis* (MTB) and drug resistance, although their sensitivity in children is suboptimal compared to culture, ranging from 62% to 80%.[9,10]

To overcome these limitations, researchers have evaluated various blood biomarkers, including cytokine profiles,[11—13] cell surface biomarkers,[14,15] antibodies,[16,17] mRNA transcript signatures,[18,19] circulating miRNAs,[20,21] and plasma metabolites[22,23] in an effort to improve the diagnosis of pediatric TB. However, the vast majority of reports are based on early-stage studies, with highly diverse designs and methodologies, and virtually all promising biomarkers that have been identified to date require further validation in separate, larger pediatric cohorts.

The aims of this study were to evaluate the diagnostic potential of 17 biomarkers in unstimulated plasma in children, including cytokines, chemokines, and markers of tissue damage, and to determine whether analysis of these biomarkers in isolation or in combination can be exploited to identify TB infection and to discriminate between LTBI and active TB.

Materials and methods

Study population

Children younger than 16 years of age evaluated for TB infection between January 2015 and March 2016 were enrolled prospectively at a large tertiary pediatric hospital in Madrid, Spain. The inclusion criteria comprised recent migration from a TB-endemic region, known contact with a TB case, or clinical/radiologic signs or

symptoms indicative of TB disease. Children with known immunodeficiency or receiving immunosuppressive medication were excluded.

The study participants underwent a physical examination, a tuberculin skin test (TST; intradermal injection of 0.1 mL of purified protein derivative PPD RT23; Statens Serum Institut, Copenhagen, Denmark) and an IGRA (QuantiFERON-TB Gold In-Tube assay; Cellestis/Qiagen, Hilden, Germany). According to local guidelines, the TST was considered positive if there was an induration $\geq$10 mm in children evaluated during TB screening and $\geq$5 mm in children who had known contact with a TB case or clinical or radiologic signs/symptoms suggestive of TB.[24] Children with a positive TST or IGRA result, as well as those who had clinical signs or symptoms suggestive of TB or had been exposed to TB within the past 2 months, also underwent a chest x-ray. Microbiological tests, including sputum smear microscopy, culture, and Xpert MTB/RIF assays, were performed in children suspected of having TB disease.

Classification of diagnostic groups

Study participants were classified into 4 groups: 1) Confirmed TB, i.e., MTB detected by culture or polymerase chain reaction and clinical signs/symptoms or radiological findings consistent with TB disease; 2) Probable TB, i.e., clinical signs/symptoms or radiological findings consistent with TB disease in a child without microbiological confirmation and at least 1 of i) a favorable response to anti-TB therapy, ii) a positive TST/IGRA result, or iii) epidemiological risk factors for TB infection; 3) Latent TB infection (LTBI), i.e., a positive TST or IGRA result without clinical symptoms, normal examination, and an unremarkable chest x-ray; 4) non—MTB-infected, i.e., an asymptomatic child with negative TST and IGRA results and an unremarkable chest x-ray, if performed.

In this report, the term "active TB" refers to patients with confirmed TB and probable TB, while "MTB infection" refers to children with confirmed TB, probable TB, and LTBI.

Biomarker assays

After enrolment, venous blood was collected from study participants in 5 mL sodium heparin tubes, and plasma was separated by centrifugation and stored at -80 °C. Plasma samples were later analyzed for the presence of cytokines/chemokines (CXCL11, IFN-γ, IL1-ra, IL-2, IL-9, IL-10, IL-13, IL-21, IP-10, MCP-1, MCP-3, MDC, MIP-1β, and TNF-α) and matrix metalloproteinases (MMP-1, MMP-7, and MMP-8) using ProcartaPlex multiplex immunoassays (Affymetrix eBioscience, Hatfield, UK). Magnetic beads were aliquoted in 96-well plates followed by the addition of standards and plasma. After an incubation period, plates were washed using a magnetic wash station, with the addition of detection antibodies. Streptavidin—phycoerythrin was added to each well, and plates were read twice using a Luminex 200 instrument (Luminex Corporation, Austin, TX, USA), and Bio-Plex Manager 6.1 software (Bio-Rad, Hercules, CA, USA).

Statistical analysis

Categorical variables are presented as frequency and percentage; continuous variables are presented as median and interquartile range (IQR). The Mann-Whitney test or Kruskal-Wallis test was used, as appropriate, to compare plasma biomarker concentrations across study groups. The comparisons were adjusted with the Benjamini-Hochberg method to control the False Discovery Rate (FDR). The correlation between plasma biomarkers in both non—MTB-infected and MTB-infected patients was explored using the Spearman correlation test. For the most relevant cytokines identified, we explored the potential of using cytokine combinations and ratios to improve diagnostic performance. To evaluate the diagnostic accuracy of plasma biomarkers in identifying MTB infection, we constructed receiver operating characteristic (ROC) curves and calculated the area under the curve (AUC), sensitivity, and specificity at the optimal cut-off based on Youden's index. IBM SPSS for Windows, Version 28.0.1.1 (IBM Corp., Armonk, NY, USA) was used for all data analyses, and 2-sided p values < 0.05 were considered statistically significant.

Ethics statement

The study was conducted in accordance with the principles of the Declaration of Helsinki and approved by the Clinical Research Ethics Committee of Hospital General Universitario Gregorio Marañón. Written informed consent was obtained from the parents/guardians of all participants prior to inclusion into the study.

Results

Demographic characteristics of study participants

The study included a total of 48 children (15 healthy controls and 33 with MTB infection). The MTB-infected group comprised 8 children with LTBI, 19 with probable TB, and 6 with confirmed TB. The median age of the participants was 4.5 years (IQR: 3.3—5.3), and most were born in Spain. Only 1 child was BCG-vaccinated. The participants' characteristics are summarized in Table 1.

Plasma biomarker profile at different stages of MTB infection

The analysis of plasma biomarkers identified significant differences between the diagnostic groups in the concentrations of IFN-γ, IL-2, and MCP-1, as well as in the IFN-γ/MCP-1 and IL-2/MCP-1 ratios (Table 2). These findings persisted after adjustment for multiple comparisons, except for MCP-1, which however showed a strong trend towards significance. However, no significant differences were observed between the groups for other biomarkers, including CXCL-11, IL-1ra, IL-9, IL-10, IL-13, IL-21, IP-10, MCP-3, MDC, MIP-1β, MMP-1, MMP-7, MMP-8, and TNF-α.

Additional statistical comparisons between diagnostic groups revealed that patients with confirmed TB had significantly higher levels of IFN-γ and IL-2 than non—MTB-

Table 1 Baseline characteristics of study participants and immunological and radiological test results at recruitment, according to diagnostic subgroups.

		Non–MTB-infected (n = 15)	MTB-infected (n = 33)		
			Latent TB (n = 8)	Probable TB (n = 19)	Confirmed TB (n = 6)
Female sex		8 (53.3)	5 (62.5)	5 (26.3)	2 (33.3)
Median age (years)		5.1 [3.9–5.7]	4.9 [3.1–5.6]	4.1 [3.6–5.3]	4.4 [2.8–7.7]
Country of birth	Spain	14 (93.3)	8 (100.0)	19 (100.0)	6 (100.0)
	Other country	1 (6.7)	0 (0.0)	0 (0.0)	0 (0.0)
Parents' country of birth	Spain	12 (80.0)	8 (100.0)	18 (94.7)	3 (50.0)
	Other country	3 (20.0)	0 (0.0)	1 (5.3)	3 (50.0)
BCG vaccination status	Vaccinated	1 (6.7)	0 (0.0)	0 (0.0)	0 (0.0)
	Unvaccinated	14 (93.3)	8 (100.0)	19 (100.0)	6 (100.0)
Tuberculin skin test	Positive	0 (0.0)	8 (100.0)	19 (100.0)	6 (100.0)
	Negative	0 (0.0)	0 (0.0)	0 (0.0)	0 (0.0)
	Induration (mm [IQR])	0 [0–0]	10 [8–15]	19 [16–20]	17 [12–22]
QuantiFERON-TB assay	Positive	0 (0.0)	1 (12.5)	17 (89.5)	6 (100.0)
	Negative	15 (100.0)	7 (87.5)[a]	2 (10.5)	0 (0.0)
	Indeterminate	0 (0.0)	0 (0.0)	0 (0.0)	0 (0.0)
Chest x-ray	Normal	15 (100.0)	8 (100.0)	0 (0.0)	1 (16.7)[b]
	Findings suggestive of TB	0 (0.0)	0 (0.0)	19 (100.0)	5 (83.3)

[a] All 7 patients had a positive tuberculin skin test and a negative QuantiFERON-TB assay result; the patients had not received the BCG vaccination and had no prior history of mycobacterial infections.
[b] The chest x-ray was normal in 1 child with microbiologically confirmed TB lymphadenitis. Abbreviations: MTB, *Mycobacterium tuberculosis*; TB, tuberculosis; IQR, interquartile range; BCG, bacille Calmette-Guérin.
Data are presented as number of patients (percentage) or median value [IQR].

Table 2 Plasma biomarker concentrations and selected ratios in the 4 diagnostic subgroups.

	Non–MTB infected (n = 15)	Latent TB (n = 8)	Probable TB (n = 19)	Confirmed TB (n = 6)	p-value	adjusted p-value
Biomarkers						
IFN-γ	**1.45 (1.36–1.71)**	**1.49 (1.27–1.80)**	**1.54 (1.36–2.32)**	**3.09 (2.41–4.18)**	**0.008**	**0.038**
TNF-α	4.32 (3.75–4.60)	3.74 (3.01–4.60)	3.75 (3.46–4.17)	4.88 (4.03–5.58)	0.141	0.446
IL-1ra	147.02 (67.67–255.93)	147.02 (88.92–280.81)	129.10 (67.67–334.61)	179.51 (67.67–241.72)	0.997	0.997
IL-2	**2.13* (2.13*-2.13*)**	**2.13* (2.13*-2.13*)**	**2.13* (2.13*-2.13)**	**6.33 (2.13*-11.7)**	**0.003**	**0.038**
IL-9	0.08* (0.08*-0.51)	0.08* (0.08*-0.08*)	0.08* (0.08*-0.68)	1.32 (0.08*-2.56)	0.328	0.566
IL-10	0.91 (0.80–1.17)	0.98 (0.82–1.26)	0.97 (0.85–1.19)	1.20 (0.66–1.74)	0.946	0.997
IL-13	0.74 (0.74–0.90)	0.77 (0.68–0.96)	0.85 (0.74–1.08)	1.12 (0.74–2.23)	0.358	0.566
IL-21	0.20* (0.20*-0.20*)	0.20* (0.20*-0.20*)	0.20* (0.20*-0.20*)	0.20* (0.20*-1.93)	0.107	0.566
IP-10	11.68 (9.40–22.39)	12.80 (8.30–17.16)	15.52 (11.91–22.87)	18.38 (11.41–20.66)	0.492	0.653
CXCL-11	5.14* (5.14*-5.14*)	5.14* (5.14*-5.14*)	5.14* (5.14*-5.14*)	5.14* (5.14*46.62)	0.355	0.566
MCP-1	**16.70 (11.34–25.79)**	**9.35 (5.04–11.21)**	**9.68 (6.83–12.30)**	**13.66 (8.23–26.68)**	**0.018**	0.068
MCP-3	5.29* (5.29*-5.29*)	5.29* (5.29*-6.8)	5.29* (5.29*-12.64)	5.29* (5.29*-12.64)	0.766	0.856
MDC	68.94 (54.67–76.07)	69.88 (68.61–89.54)	75.60 (48.77–88.84)	52.32 (35.34–57.44)	0.434	0.634
MIP-1β	5.69* (5.69*-5.69*)	5.69* (5.69*-5.69*)	5.69* (5.69*-5.69*)	5.69* (5.69*-33.27)	0.331	0.566
MMP-1	8.32 (4.21–8.32)	5.61 (4.21–6.96)	5.61 (2.81–8.32)	14.18 (5.61–17.38)	0.177	0.480
MMP-7	186.30 (149.19–323.85)	177.30 (94.4–252.49)	140.64 (100.35–209.69)	223.93 (47.07–232.25)	0.582	0.691
MMP-8	51.60 (27.08–107.74)	26.17 (23.14–68.68)	37.22 (21.94–68.48)	52.24 (30.44–79.10)	0.516	0.653
Biomarker ratios						
IFN-γ/MCP-1	**0.08 (0.06–0.15)**	**0.16 (0.11–0.33)**	**0.20 (0.13–0.29)**	**0.28 (0.11–0.65)**	**0.004**	**0.038**
IL-2/MCP-1	**0.13 (0.10–0.22)**	**0.22 (0.19–0.42)**	**0.23 (0.17–0.38)**	**0.43 (0.25–1.64)**	**0.008**	**0.038**

All cytokine data from every participant were included (n = 48), with the exception of MCP-1 (n = 45) and IL-2 (n = 47), as there were reading errors in 3 and 1 patients, respectively. Cytokine concentrations are shown in pg/ml. Data are expressed as median and interquartile range. p-Values are based on the Kruskal-Wallis test. Adjusted p-values correspond to the Benjamini-Hochberg correction for multiple comparisons. Significant values (<0.05) are shown in bold. Values below the assay's range of detection were corrected with the minimal concentration observed (*). Abbreviations: MTB, *Mycobacterium tuberculosis*; TB, tuberculosis.

infected children and those with LTBI (Table 3). Conversely, MCP-1 levels were significantly lower in patients with LTBI and probable TB than in non–MTB-infected children. Furthermore, the IFN-γ/MCP-1 and IL-2/MCP-1 ratios were higher in all 3 MTB-infected groups than in non–MTB-infected children (Fig. 1). Overall, our results remained significant after adjustment, with the exception of MCP-1, IFN-γ/MCP-1, and IL-2/MCP-1 ratios, which approached significance, indicating potential biological relevance.

Further pairwise comparisons were conducted between non–MTB-infected and MTB-infected children (comprising LTBI, probable TB, and confirmed TB), revealing significantly lower concentrations of MCP-1 ($p = 0.004$) and higher ratios of IFN-γ/MCP-1 and IL-2/MCP-1 in MTB-infected children ($p < 0.0001$ and $p = 0.001$, respectively; see Supplementary Fig. 1).

Correlation analysis of selected plasma biomarkers

The correlation analysis demonstrated significant correlations between certain biomarkers in all diagnostic groups (Fig. 2 & Supplementary Fig. 2). The strongest correlations were observed in children with confirmed TB, where IFN-γ correlated well with IL-10 ($r = 0.95$; $p < 0.001$), while IL-2 correlated well with IL-1ra ($r = 0.92$, $p < 0.01$), IL-21 ($r = 0.91$; $p < 0.01$), MCP-3 ($r = 0.84$, $p < 0.04$), and MMP-1 ($r = 0.85$, $p = 0.03$). In the subgroup of children with MTB infection, a moderate correlation was identified between IFN-γ and IL-2 ($r = 0.7$, $p < 0.001$) and IL-10 ($r = 0.63$, $p < 0.001$). A moderate correlation was also identified between IL-2 and TNF-α ($r = 0.7$, $p < 0.001$) and MMP-1 ($r = 0.8$, $p < 0.001$).

Diagnostic accuracy for identifying MTB infection

ROC curve analyses were conducted to evaluate the accuracy of the biomarkers previously identified to have potential discriminatory ability. The IFN-γ/MCP-1 ratio was the most accurate parameter for distinguishing between non–MTB-infected and MTB-infected children (AUC 0.82, sensitivity 87.9%, specificity 66.6%, $p < 0.001$), as well as between non–MTB-infected children and those with active TB (AUC 0.82, sensitivity 88.0%, specificity 60%, $p < 0.001$). The IL-2/MCP-1 ratio also displayed good discriminatory ability in both comparisons. However, none of the individual biomarkers or ratios thereof were able to distinguish between LTBI and active TB. The relevant data are summarized in Supplementary Table 1 and Fig. 3.

Discussion

In this study, we evaluated 17 plasma biomarkers from well-defined patient groups, namely, non–MTB-infected children, children with LTBI, and children with TB disease. Our findings indicate that none of the biomarkers alone evaluated in unstimulated blood samples enables a robust distinction between MTB-infected and non–MTB-infected individuals. However, by combining cytokine biomarkers the diagnostic performance was enhanced to levels close to the WHO target product profile for a TB triage test. Based on expert consultation, the WHO target product profile

Table 3 Comparison of selected plasma cytokines and cytokine ratios in the study subgroups.

Biomarker	Four-group comparison p-value *(adjusted p-value)*	Pairwise comparison p-value (adjusted p-value)				
		Non–MTB-infected *vs* LTBI	Non–MTB-infected *vs* Probable TB	Non–MTB-infected *vs* Confirmed TB	LTBI *vs* Probable TB	LTBI *vs* Confirmed TB	Probable TB *vs* Confirmed TB
IFN-U	**0.008 (0.038)**	0.996 (1)	0.212 (0.543)	**0.001 (0.009)**	0.305 (0.938)	**0.003 (0.028)**	**0.015** (0.145)
IL-2	**0.004 (0.038)**	0.664 (1)	0.716 (0.906)	**0.001 (0.009)**	0.454 (0.938)	**0.001 (0.019)**	**0.001 (0.019)**
MCP-1	**0.018** (0.068)	**0.006** (0.114)	**0.009** (0.080)	0.470 (0.811)	0.474 (0.938)	0.155 (0.490)	0.319 (0.710)
IFN-U/MCP-1	**0.002 (0.038)**	**0.022** (0.196)	**0.001 (0.019)**	**0.003 (0.014)**	0.807 (0.938)	0.425 (0.621)	0.484 (0.710)
IL-2/MCP-1	**0.008 (0.038)**	**0.031** (0.196)	0.017 (0.107)	**0.002 (0.012)**	0.782 (0.938)	0.319 (0.595)	0.162 (0.615)

Statistical significance was analyzed using the Kruskal-Wallis test (4 group comparisons) or the Mann-Whitney test (2 pairwise comparisons); significant values (<0.05) are shown in bold. Adjusted p-values correspond to the Benjamini-Hochberg correction for multiple comparisons. All cytokine data from each participant were included ($n = 48$), with the exception of MCP-1 ($n = 45$) and IL-2 ($n = 47$), which were subject to reading errors in 3 and 1 patients, respectively. Abbreviations: LTBI, latent tuberculosis infection; MTB, *Mycobacterium tuberculosis*; TB, tuberculosis.

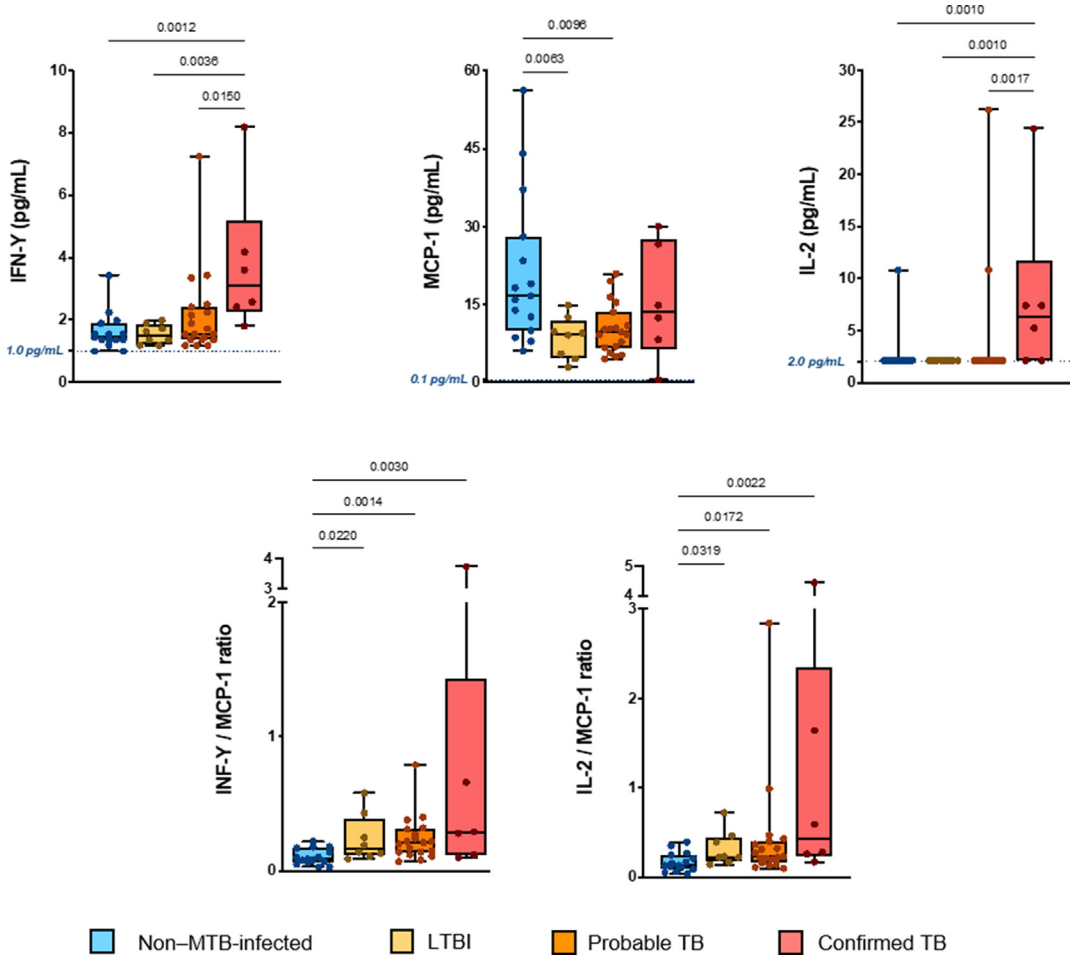

Fig. 1. Plasma biomarker concentrations in the diagnostic subgroups. Box-whisker plots show cytokine concentrations or ratios. Statistical significance was tested using using the Mann-Whitney test (pairwise comparisons). $P < 0.05$ was considered statistically significant; all statistically significant comparisons are shown. The cytokine concentration cut-off for IFN-Y, MCP-1, and IL-2 is depicted in blue. Abbreviations: TB, tuberculosis; LTBI, latent TB infection.

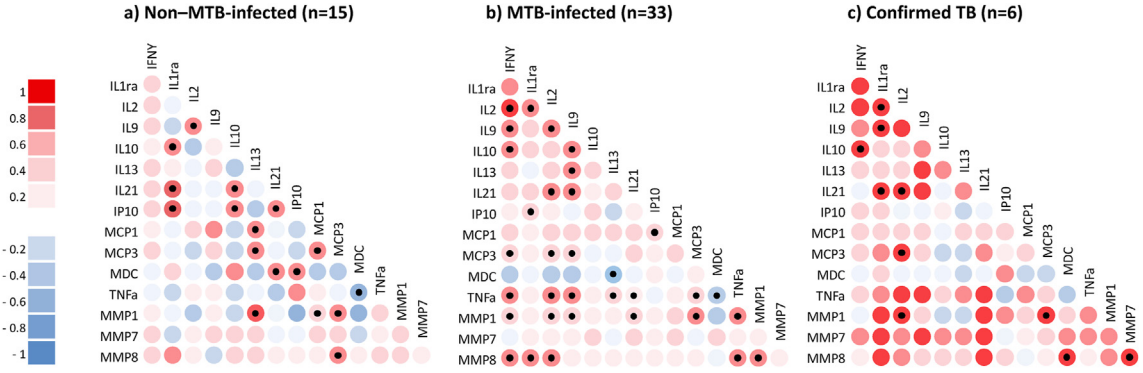

Fig. 2. Correlation analysis of plasma biomarkers Correlogram showing blood biomarkers in plasma in a) Non—MTB-infected, b) MTB-infected, and c) confirmed TB groups. The scale represents the Spearman rho value; red circles correspond to the maximum positive correlation and blue circles to the maximum negative correlation. Black dots indicate statistical significance. Correlations of CXCL11 and MIP1beta with other biomarkers are omitted due to constant values in several study groups. $P < 0.05$ was considered statistically significant. Abbreviations: MTB, *Mycobacterium tuberculosis*.

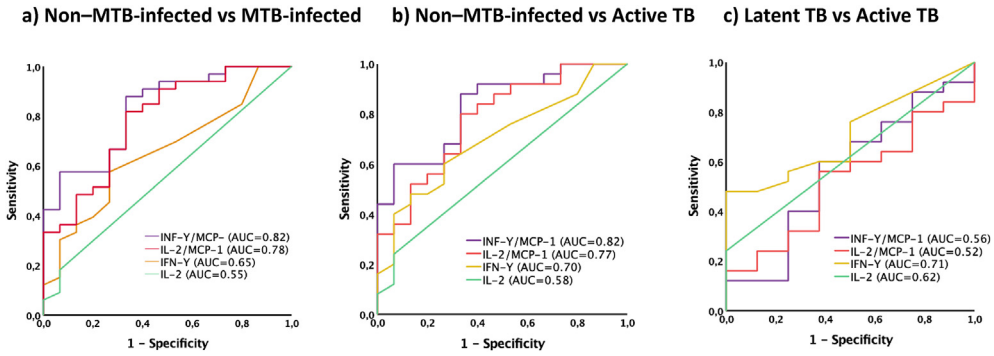

Fig. 3. Receiver operating characteristic (ROC) curves of individual cytokine biomarkers with potential discriminatory ability and ratios thereof. a) Non-MTB-infected vs MTB-infected; b) Non-MTB-infected vs Active TB; c) Latent TB vs Active TB. Analysis conducted in children without MTB infection, children with active TB (comprising patients with probable TB and confirmed TB), and patients with latent TB.

suggests 90% test sensitivity in combination with >70% test specificity as a minimum requirement. The best performing biomarker combinations in our study were the IFN-γ/MCP-1 and IL-2/MCP-1 ratios, with the former achieving 87.9% sensitivity and 66.6% specificity.

Many studies have characterized the complex interplay between MTB and human innate and acquired immune responses, revealing that LTBI and TB disease represent different stages of host—pathogen interactions along a continuum, rather than distinct infection states.[25—27] The human immune response to MTB encompasses alveolar macrophages, $CD4^+$ T cells, $CD8^+$ T cells, CD1b-restricted T cells and NKT cells, and release of a range of pro-inflammatory and anti-inflammatory cytokines, as well as chemokines.[28—30] Pro-inflammatory cytokines, including IL-2, IL-9, IL-21, IFN-γ, and TNF-α, promote the activation of macrophages and recruitment of additional immune cells to the site of infection. Anti-inflammatory cytokines such as IL-10, IL-13, and TGF-β modulate the cellular immune response, preventing excessive inflammation and tissue damage. Chemokines, such as CXCL11, IP-10, MCP-1, and MDC, play a critical role in the chemotaxis of cell populations towards lung granuloma.[31]

Many studies have explored the potential of serum cytokines and chemokines to be used for diagnostic purposes, although to date, IGRA, which is based on the detection of IFN-γ following stimulation with mycobacterial peptides, remains the only immune-based test for MTB infection used in clinical practice. Nevertheless, IGRA requires incubation for 16—24 h, followed by ELISA, which typically results in a 2- to 3-day delay in the assay result becoming available to the treating clinician. In contrast, with an appropriate laboratory set-up, analysis of unstimulated serum samples could yield a result the same day.

To date there remains an urgent need for a TB test that is accurate and inexpensive and can be performed in both high- and low-infrastructure settings. Most blood biomarker assays, including commercial IGRAs, need complex laboratory infrastructure to be implemented. In our study, we explored blood biomarkers in unstimulated plasma, which is easy to obtain and process and could form the basis point-of-care or near-patient testing. As highlighted in a comprehensive review on this topic, serial measurement of certain serum cytokines in unstimulated blood samples, including IL-6, IL-10, and TNF-α, could also provide useful information regarding response to treatment.[12]

In our study, IFN-γ, IL-2, and MCP-1 were the biomarkers most able to discriminate between healthy controls and children at different stages of MTB infection, underscoring the significant role of those cytokines in disease containment and progression of MTB infection. Our findings are broadly in accordance with those of previous studies using unstimulated plasma samples. One study in adult patients in China found that IFN-γ and MCP-1 concentrations were significantly higher in study participants with LTBI and TB disease than in noninfected controls.[32] Furthermore, a study in adults in Taiwan found that IFN-γ and MCP-1 concentrations were significantly higher in patients with pulmonary TB than in healthy controls.[33] Finally, a Dutch study that included both adolescents and adults, of whom 45 had pulmonary TB and 36 extrapulmonary TB, found that serum IFN-γ concentrations were significantly higher in patients with TB disease than in healthy controls and that those elevated concentrations subsequently normalized during anti-TB therapy.[34]

With a view to improving the performance of the biomarkers identified, we explored the use of cytokine ratios, which we found to be more sensitive and specific than any single biomarker evaluated in this study. Several studies have assessed the potential of using combined biomarkers to discriminate between TB infection stages. A study from The Gambia performed using blood stimulated with mycobacterial antigens reported that a combination of IL-12 (p40), IL-17, and TNF-α was able to correctly classify 79% of the study participants as having LTBI or TB disease.[35] Another large-scale study, which was performed using unstimulated serum samples with recruitment sites in several African countries, found that combining IFN-γ with IP-10 and 5 non-specific markers of inflammation (apolipoprotein A-1, complement factor H, C-reactive protein, serum amyloid A, and transthyretin) enabled TB disease to be diagnosed with high sensitivity (94%), although the specificity (73%) of this combination was suboptimal.[36]

Several adult studies have explored the use of matrix metalloproteinases (MMPs), a family of proteolytic enzymes that are implicated in tissue damage in TB disease, as blood

biomarkers of TB disease. However, very few have evaluated MMPs in children.[37] Adult studies have consistently shown that serum MMP-1 and MMP-8 concentrations are higher in pulmonary TB cases than in healthy and in sick controls; in contrast, observations regarding MMP-2, MMP-3, MMP-7, and MMP-9 have been conflicting.[38] A pediatric study in Brazil that included 14 children with pulmonary TB and 22 with extra-pulmonary TB, found that serum MMP-7 concentrations were significantly higher in the pulmonary TB group than in healthy controls, although this was not observed in extra-pulmonary TB patients.[39] Similarly, a recently published South African study on children with TB meningitis, a severe form of extra-pulmonary TB, did not observe any differences in serum MMP concentrations (MMP-1, MMP-7, MMP-8, and MMP-9) between cases of TB meningitis and children with other forms of meningitis.[38] Our univariable analysis did not reveal a differential pattern for MMPs across the study groups. However, the analysis exploring associations between different biomarkers revealed that in patients with confirmed TB, there was a moderate-to-strong positive correlation between MMP-7 and several cytokines that play a key role in the human immune response to MTB, including IFN-γ, IL-1ra, IL-2, IL-10, and IL-13.

Our study has several limitations. First, the number of participants included in each group was comparatively small, particularly in the LTBI and confirmed TB groups. Nevertheless, we were able to detect significant differences in the concentrations of key cytokines between the diagnostic groups. Second, in common with other pediatric TB studies, microbiological confirmation was not available for a large proportion of children diagnosed with TB disease; this is unavoidable due to the paucibacillary nature of TB disease in young children. In addition, there is no microbiological gold standard for LTBI, and test results were discordant (TST+/IGRA-) in many of the patients in this group. Nevertheless, since none of the children with this result had been vaccinated with BCG or had a previous history of TB, it is likely that they were truly MTB-infected, rather than having false-positive TST results. Another limitation of the study is that none of the biomarkers were able to distinguish between LTBI and active disease. Finally, after adjusting for multiple comparisons, certain cytokines lost significance but showed strong trends towards it, indicating the need for further research with larger sample sizes to confirm these trends and their biological relevance.

In conclusion, our study suggests that plasma biomarkers could serve as a complementary diagnostic tool for TB in children. Among the biomarkers and biomarker combinations evaluated in this study, the IFN-γ/MCP-1 ratio performed best for differentiating between MTB-infected and non–MTB-infected children. However, the results of this study will require further validation in independent, larger cohorts of children and adolescents.

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CRediT authorship contribution statement

Andrea López-Suárez: Formal analysis, Investigation, Resources, Writing — original draft, Writing — review & editing. **Mar Santos-Sebastián:** Investigation, Resources. **Alicia Hernanz-Lobo:** Investigation, Resources. **Elena Rincón-López:** Investigation, Resources. **David Aguilera-Alonso:** Investigation, Resources. **Jesús Saavedra-Lozano:** Investigation, Resources. **María Jesús Ruiz Serrano:** Investigation, Methodology, Resources. **Ángel Hernández-Bartolomé:** Investigation, Validation, Writing — original draft, Writing — review & editing. **Luz María Medrano de Dios:** Investigation, Methodology. **José Luis Jiménez Fuentes:** Investigation, Validation. **María Luisa Navarro:** Investigation, Resources. **Marc Tebruegge:** Conceptualization, Data curation, Investigation, Supervision, Visualization, Writing — review & editing. **Begoña Santiago-García:** Conceptualization, Data curation, Formal analysis, Funding acquisition, Supervision, Visualization, Writing — original draft, Writing — review & editing.

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References

1. World Health Organization. Global tuberculosis report 2023. Published online 2023.
2. TB/COVID-19 Global Study Group. Tuberculosis and COVID-19 co-infection: description of the global cohort. *Eur Respir J* 2022;Mar 24;**59**(3).
3. Keshavjee S, Amanullah F, Cattamanchi A, et al. Moving toward tuberculosis elimination critical issues for research in diagnostics and therapeutics for tuberculosis infection. *Am J Respir Crit Care Med* 2019;**199**(5):564–71. https://doi.org/10.1164/rccm.201806-1053PP.
4. Vaezipour N, Fritschi N, Brasier N, et al. Towards accurate point-of-care tests for tuberculosis in children. *Pathogens* 2022;**11**(3). https://doi.org/10.3390/pathogens11030327.
5. Kay AW, Islam SM, Wendorf K, Westenhouse J, Barry PM. Interferon-γ release assay performance for tuberculosis in childhood. *Pediatrics* 2018;Jun;**141**(6):e201739918.
6. Buonsenso D, Noguera-Julian A, Moroni R, et al. Performance of QuantiFERON-TB Gold Plus assays in paediatric tuberculosis: a multicentre PTBNET study. *Thorax* 2023;**78**:288–96. https://doi.org/10.1136/thoraxjnl-2022-218929.
7. Tebruegge M, De Graaf H, Sukhtankar P, et al. Extremes of age are associated with indeterminate QuantiFERON-TB gold assay results. *J Clin Microbiol* 2014;**52**(7):2694–7. https://doi.org/10.1128/JCM.00814-14.
8. Tebruegge M, Ritz N, Curtis N, Shingadia D. Diagnostic tests for childhood tuberculosis: past imperfect, present tense, future

perfect. *Pediatr Infect Dis J* 2015;**34**(9):1014—9. https://doi.org/10.1097/INF.0000000000000796.
9. Detjen AK, DiNardo AR, Leyden J, et al. Xpert MTB/RIF assay for the diagnosis of pulmonary tuberculosis in children: a systematic review and meta-analysis. *Lancet Respir Med* 2015;**3**(6):451—61. https://doi.org/10.1016/S2213-2600(15)00095-8.
10. Aguilera-Alonso D, Solís-García G, Noguera-Julian A, et al. Accuracy of Xpert Ultra for the diagnosis of paediatric tuberculosis in a low TB burden country: a prospective multicentre study Tuberculosis. *Thorax* 2022;**77**:1023—9. https://doi.org/10.1136/thoraxjnl-2021-218378.
11. Togun TO, MacLean E, Kampmann B, Pai M. Biomarkers for diagnosis of childhood tuberculosis: a systematic review. *PLoS One* 2018;**13**(9):1—19. https://doi.org/10.1371/journal.pone.0204029.
12. Clifford V, Zufferey C, Street A, Denholm J, Tebruegge M, Curtis N. Cytokines for monitoring anti-tuberculous therapy: a systematic review. *Tuberculosis* 2015;**95**(3):217—28. https://doi.org/10.1016/j.tube.2015.01.003.
13. Sudbury EL, Otero L, Tebruegge M, et al. Mycobacterium tuberculosis-specific cytokine biomarkers for the diagnosis of childhood TB in a TB-endemic setting. *J Clin Tuberc Other Mycobact Dis* 2019;**16**:100102. https://doi.org/10.1016/j.jctube.2019.100102.
14. Portevin D, Moukambi F, Clowes P, et al. Assessment of the novel T-cell activation marker — tuberculosis assay for diagnosis of active tuberculosis in children : a prospective proof-of-concept study. *Lancet Infect Dis* 2014;**14**:931—8. https://doi.org/10.1016/S1473-3099(14)70884-9.
15. Ahmed MIM, Ziegler C, Held K, et al. The TAM-TB assay-A promising TB immune-diagnostic test with a potential for treatment monitoring. *Front Pediatr* 2019;**7**(FEB). https://doi.org/10.3389/fped.2019.00027.
16. Lu LL, Das J, Grace PS, Fortune SM, Restrepo BI, Alter G. Antibody Fc glycosylation discriminates between latent and active tuberculosis. *JID (J Infect Dis)* 2020;**222**(12):2093—102. https://doi.org/10.1093/infdis/jiz643.
17. Lu LL, Chung AW, Rosebrock TR, et al. A functional role for antibodies in tuberculosis. *Cell* 2016;**167**(2):433—443.e14. https://doi.org/10.1016/j.cell.2016.08.072.
18. Anderson ST, Kaforou M, Brent AJ, et al. Diagnosis of childhood tuberculosis and host RNA expression in africa. *N Engl J Med* 2014;**370**(18):1712—23. https://doi.org/10.1056/nejmoa1303657.
19. Warsinske H, Vashisht R, Khatri P. Host-response-based gene signatures for tuberculosis diagnosis: a systematic comparison of 16 signatures. *PLoS Med* 2019;**16**(4). https://doi.org/10.1371/journal.pmed.1002786.
20. Wang JX, Xu J, Han YF, Zhu YB, Zhang WJ. Diagnostic values of microRNA-31 in peripheral blood mononuclear cells for pediatric pulmonary tuberculosis in Chinese patients. *Genet Mol Res* 2015;**14**(4):17235—43. https://doi.org/10.4238/2015.December.16.23.
21. Zhou M, Yu G, Yang X, Zhu C, Zhang Z, Zhan X. Circulating microRNAs as biomarkers for the early diagnosis of childhood tuberculosis infection. *Mol Med Rep* 2016;**13**(6):4620—6. https://doi.org/10.3892/mmr.2016.5097.
22. Andreas NJ, Basu Roy R, Gomez-Romero M, et al. Performance of metabonomic serum analysis for diagnostics in paediatric tuberculosis. *Sci Rep* 2020;**10**(1). https://doi.org/10.1038/s41598-020-64413-6.
23. Sun L, Li JQ, Ren N, et al. Utility of novel plasma metabolic markers in the diagnosis of pediatric tuberculosis: a classification and regression tree analysis approach. *J Proteome Res* 2016;**15**(9):3118—25. https://doi.org/10.1021/acs.jproteome.6b00228.
24. Baquero-Artigao F, del Rosal T, Falcón-Neyra L, et al. Actualización del diagnóstico y tratamiento de la tuberculosis. *An Pediatr* 2023;**98**:460—9. https://doi.org/10.1016/j.anpedi.2023.03.011.
25. de Martino M, Lodi L, Galli L, Chiappini E. Immune response to Mycobacterium tuberculosis: a narrative review. *Front Pediatr* 2019;**7**(August):1—8. https://doi.org/10.3389/fped.2019.00350.
26. Domingo-Gonzalez R, Prince O, Cooper A, Khader SA. Cytokines and chemokines in Mycobacterium tuberculosis infection. *Microbiol Spectr* 2016;Oct;(4). https://doi.org/10.1128/microbiolspec.tbtb2-0018-2016. 10.1128/microbiolspec.TBTB2-0018-2016.
27. Jee B. Understanding the early host immune response against Mycobacterium tuberculosis. *Cent Eur J Immunol* 2020;**45**(1):99—103. https://doi.org/10.5114/ceji.2020.94711.
28. Chancellor A, Tocheva AS, Cave-Ayland C, et al. CD1b-restricted GEM T cell responses are modulated by Mycobacterium tuberculosis mycolic acid meromycolate chains. *Proc Natl Acad Sci USA* 2017;**114**(51):E10956—64. https://doi.org/10.1073/pnas.1708252114.
29. Tebruegge M, Ritz N, Donath S, et al. Mycobacteria-specific mono- and polyfunctional CD4+ T cell profiles in children with latent and active tuberculosis: a prospective proof-of-concept study. *Front Immunol* 2019;**10**(APR). https://doi.org/10.3389/fimmu.2019.00431.
30. Kaufmann SHE, Fortune S, Pepponi I, Ruhwald M, Schrager LK, Ottenhoff THM. TB biomarkers, TB correlates and human challenge models: new tools for improving assessment of new TB vaccines. *Tuberculosis* 2016;**99**:S8—11. https://doi.org/10.1016/j.tube.2016.05.010.
31. Tezera LB, Bielecka MK, Chancellor A, et al. Dissection of the host-pathogen interaction in human tuberculosis using a bioengineered 3-dimensional model. *Elife* 2017;**6**. https://doi.org/10.7554/eLife.21283.
32. Wang S, Li Y, Shen Y, et al. Screening and identification of a six-cytokine biosignature for detecting TB infection and discriminating active from latent TB. *J Transl Med* 2018;**16**(1):1—13. https://doi.org/10.1186/s12967-018-1572-x.
33. Su WL, Perng WC, Huang CH, Yang CY, Wu CP, Chen JH. Association of reduced tumor necrosis factor alpha, gamma interferon, and interleukin-1β (IL-1β) but increased IL-10 expression with improved chest radiography in patients with pulmonary tuberculosis. *Clin Vaccine Immunol* 2010;**17**(2):223—31. https://doi.org/10.1128/CVI.00381-09.
34. Verbon A, Juffermans N, Van Deventer SJH, Speelman P, Van Deutekom H, Van Der Poll T. Serum concentrations of cytokines in patients with active tuberculosis (TB) and after treatment. *Clin Exp Immunol* 1999;**115**(1):110—3.
35. Sutherland JS, Jong BC De, Jeffries DJ, Adetifa IM, Ota MOC. Production of TNF- a , IL-12 (p40) and IL-17 can discriminate between active TB disease and latent infection in a west african cohort. *PLoS One* 2010;**5**(8):e12365. https://doi.org/10.1371/journal.pone.0012365.
36. Chegou NN, Sutherland JS, Malherbe S, et al. Diagnostic performance of a seven-marker serum protein biosignature for the diagnosis of active TB disease in African primary healthcare clinic attendees with signs and symptoms suggestive of TB. *Thorax* 2016;**71**(9):785—94. https://doi.org/10.1136/thoraxjnl-2015-207999.
37. Rohlwink UK, Walker NF, Ordonez AA, et al. Matrix metalloproteinases in pulmonary and central nervous system tuberculosis—a review. *Int J Mol Sci* 2019;**20**(6). https://doi.org/10.3390/ijms20061350.
38. Albuquerque VVS, Kumar NP, Fukutani KF, et al. Plasma levels of C-reactive protein, matrix metalloproteinase-7 and lipopolysaccharide-binding protein distinguish active

pulmonary or extrapulmonary tuberculosis from uninfected controls in children. *Cytokine* 2019;**123**. https://doi.org/10.1016/j.cyto.2019.154773.
39. Manyelo CM, Chegou NN, Seddon JA, et al. Serum and cerebrospinal fluid host proteins indicate stroke in children with tuberculous meningitis. *PLoS One* 2021;**16**(4 April). https://doi.org/10.1371/journal.pone.0250944.

Appendix A. Supplementary data

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