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Combination of serological and cytokine release assays for improved diagnosis of childhood tuberculosis in Zambia (PROMISE-TB)

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ABSTRACT

Objectives: The diagnostic gaps for childhood tuberculosis (TB) remain considerable in settings with high TB incidence and resource constraints. We established and evaluated the performance of a scoring system based on a combination of serological tests and T-cell cytokine release assays, chosen for their ability to detect immune responses indicative of TB, in a context of high prevalence of pediatric HIV infection.

Methods: We enrolled 628 consecutive children aged ≤ 15 years, admitted for TB suspicion. Multiple cytokine levels in QuantiFERON Gold In-Tube supernatants and antigen 85B (Ag85B) antibodies were assessed in children who tested positive with either Xpert TB or mycobacterial culture. The results were compared with those of control children.

Results: Among the biomarkers most strongly associated with TB, random forest classification analysis selected Ag85B antibodies, interleukin-2/interferon- γ ratio, and monokine induced by interferon- γ for the scoring system. The receiver operating characteristic curve derived from our scoring system showed an area under the curve of 0.95 (0.91-0.99), yielding 91% sensitivity and 88% specificity. The internal bootstrap validation gave the following 95% confidence intervals for the score performance: sensitivity 71%-97% and specificity 79%-99%.

Conclusions: This study suggests that supplementing the QuantiFERON assay with a combination of serological and T-cell markers could enhance childhood TB screening regardless of HIV status and age. Further validation among the target population is necessary to confirm the performance of this scoring system.

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Introduction

Children account for a substantial part of the tuberculosis (TB) burden, with an estimated 1 million TB cases annually and approximately 239,000 related deaths, including 88,600 in Africa and ap-

proximately 2800 in Zambia [1]. The true extent of the disease's impact on children is uncertain given the complex diagnostic nature of childhood TB. This complexity arises from the wide-ranging and non-specific symptoms observed within this age group. Obtaining good-quality sputum samples from young children poses particular challenges. Microbiological confirmation of childhood TB is generally based on gastric lavage performed during hospitalization or in outpatients, or on saline-induced sputum collection, which should be conducted by trained staff and may be difficult to perform in clinical practice. Furthermore, children are more likely to present with paucibacillary TB, which can result in a high proportion of specimens falling below the detection limits of many

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microbiological and molecular tests [2]. As a consequence, many children receive presumptive anti-TB treatment, occasionally in excess, while underdiagnosis and delayed treatment elevate the risks of severe morbidity, mortality, and TB transmission. Recognizing these challenges, the World Health Organization (WHO) has prioritized the development of biomarker-based tests that enable TB diagnosis using non-sputum samples [3].

Peripheral blood is an accessible sample for testing the immune response against TB. However, the poor performance of commercial kits led the WHO to advise against their use in 2011 because of their lack of accuracy and insufficient validation [4]. Research in serological assays, however, remains needed, as testing of serum antibodies against mycobacterial antigens would be easily measurable and accessible in the field. Serological studies using various TB antigens have reported encouraging results. In particular, antibodies directed against the antigen 85 (Ag85) complex are among the most promising biomarkers [5,6]. The clinical utility of serological tests would be further enhanced by a multistep algorithm, encompassing exposure history, clinical presentation, screening/triage tests, and confirmatory assays.

Assays based on cell-mediated immune responses following lymphocyte stimulation provide another means to explore the adaptive immune response against TB. IFN- γ (interferon- γ) release assays (IGRAs), which measure the cell-mediated immune response against the *Mycobacterium tuberculosis* RD1-encoded antigens, are widely used in low-incidence, well-resourced settings for diagnosing latent TB infection and as an adjunctive diagnostic tool in paucibacillary pulmonary TB and non-pulmonary TB. However, commercial IGRAs do not discriminate active from latent TB infection [7]. In children aged under 5 years, a positive IGRA result reflects a relatively recent *M. tuberculosis* infection with a high likelihood of progressing to TB disease [8]. Malnutrition and HIV infection are among the major risk factors for developing disease in children after infection [9,10]. In high-incidence, resource-constrained settings, the potential utility of IGRAs in the cascade of TB diagnosis in children is a subject of debate [11]. These assays do not exhibit sufficiently high performance to be considered triage tests in high-burden settings, and the cost of these assays remains a major barrier [11]. T-cell assays based on other cytokine responses might enhance TB diagnosis in adults and children. Secretion cytokines such as IP-10 (IFN- γ -induced protein 10) (also named CXCL10), TNF- α (tumor necrosis factor- α), IL-2 (interleukin-2), and MIG (monokine induced by IFN- γ) (also named CXCL9) distinctively vary between active and latent forms of TB, suggesting their potential use as diagnostic biomarkers [12].

In this proof-of-concept study, we established a scoring system based on combined immunodiagnostic tests, and assessed its efficacy for distinguishing children with confirmed TB from control children without active TB in Zambia.

Materials and methods

This study was designed and implemented according to the recommendations of the expert panel convened by the National Institutes of Health in Washington in 2011 for the evaluation of TB diagnostics in children [13,14]. Because the proposed combination of immunological tests has not been previously tested in children, we designed a proof-of-principle evaluation phase including children with confirmed active TB and healthy controls without signs of TB in a 1:2 ratio. We conducted a prospective study in a context of high prevalence of pediatric HIV infection at the University Teaching Hospital (UTH), Lusaka, Zambia and at the Arthur Davison Children Hospital, Ndola, Zambia. Consecutive children aged ≤ 15 years, hospitalized at the infectious disease units of the pediatric departments for TB suspicion, were eligible if they presented with at least two of the four TB symptoms included in the WHO

screening criteria: persistent and unexplained cough (>2 weeks); unexplained weight loss ($>5\%$ reduction in weight compared with the highest weight recorded in the last 3 months) or failure to thrive (weight for age or weight for height Z-scores < -2 and not responding to nutritional rehabilitation or to antiretroviral therapy if HIV-infected); persistent (>1 week) and unexplained fever ($>38^\circ\text{C}$); and night sweats. We aimed to recruit a similar number of HIV-infected and non-infected children, and a similar number of children aged <5 and ≥ 5 years.

The control group consisted of healthy children without any of the clinical signs mentioned above, no acute respiratory symptom, and no history of direct exposure to TB. No chest X-ray or molecular tests for TB were performed in this group. These children were selected from the reconstruction and neurosurgery wards located at UTH. Laboratory scientists were blinded to the clinical status of the children. The study was approved by the national ethical committee for medical research in Zambia, and recorded in ClinicalTrials.gov (NCT02573623).

After obtaining informed consent from the mother and/or the father, two spontaneous or induced sputum samples, gastric lavages, or adenitis needle aspiration samples, when appropriate, were collected from children in the presumptive TB group. These samples were examined for acid-fast bacilli and cultured for mycobacteria. The same samples were also used on the same day for direct smear examination for *M. tuberculosis* bacilli (MTB), MTB culture (BD BACTECTM MGITTM Automated Mycobacterial Detection System), and for molecular diagnosis using Xpert MTB/RIF (Cepheid). Children who tested positive with either Xpert TB or MTB culture were included in the group of microbiologically confirmed TB. At enrollment, 5 ml of blood was drawn, including 1 ml of whole blood in three QuantiFERON Gold In-Tube tubes (QIAGEN) incubated at 37°C . After 20 hours of incubation, all QuantiFERON supernatants were frozen at -20°C at the local European and Developing Countries Clinical Trials Partnership (EDCTP)-funded TB research laboratory at UTH, or at the Arthur Davison Children's Hospital in Ndola, and sent to the University Hospital of Montpellier for testing. According to the manufacturer's guidelines, QuantiFERON results were considered indeterminate if IFN- γ levels in the positive control tube (mitogen) and the negative control tube (Nil) were <0.5 IU/ml or >8.0 IU/ml, respectively. A serum sample was also collected and stored at -20°C for testing the presence of Ag85B antibodies at the Inserm (Institut national de la santé et de la recherche médicale) Unit 1058 in Montpellier.

Expression and purification of Ag85B

The pET23a was used to express the Ag85B protein. Briefly, a codon-optimized *fbpB* gene was obtained from GenScript and subsequently inserted into the expression plasmid using the XhoI and NdeI restriction sites, leading to the addition of a polyhistidine tag at the C-terminus of the Ag85B protein. After transformation of *Escherichia coli* C41 (DE3) and culture, the bacteria were resuspended in lysis buffer (50 mM Tris, pH 8.0, 200 mM NaCl, 20 mM imidazole, 1 mM benzamidine, 5 ml β -mercaptoethanol). Cells were broken by sonication, and the lysate was clarified by centrifugation (27,000 $\times g$, 45 minutes, 4°C) and subjected to nickel-affinity chromatography purification (Ni-NTA Sepharose, GE Healthcare Life Sciences). The eluted protein was dialyzed against a buffer (50 mM Tris-HCl, pH 8.0, 5 mM β -mercaptoethanol) at 4°C overnight and further purified by size-exclusion chromatography purification (SuperdexTM 75 10/300 GL column, GE Healthcare). The purity of the protein was assessed by SDS-PAGE (sodium dodecyl sulfate-polyacrylamide gel electrophoresis), revealing the presence of very few contaminating proteins. The recombinant Ag85B protein was eluted in potassium phosphate buffer (50 mM $\text{KH}_2\text{PO}_4/\text{K}_2\text{HPO}_4$, pH 7.6).

Immunological assays

Ag85B IgG (immunoglobulin G) enzyme-linked immunosorbent assay (ELISA)

Coating of 96-well microtiter plates (Nunc, Denmark) with 100 μ l of Ag85B at a final concentration of 2 μ g/ml was performed, followed by overnight incubation at 4°C. After washing, plates were incubated with a blocking buffer consisting of 2% bovine serum albumin (Sigma-Aldrich, USA) and 0.01% Tween 20 in phosphate-buffered saline (PBS). One hundred microliters of patient blood serum per well (dilution: 1/100 in blocking buffer) were incubated at 37°C for 1 hour. After several washing steps with PBS-Tween 20 (0.01%), bound antibodies were incubated with 100 μ l of horseradish peroxidase-conjugated mouse anti-human IgG antibody (25 ng/ml) (Antibodies-online GmbH, Germany) for an additional 1 hour at 37°C. After washing, 100 μ l of enzymatic substrate tetramethylbenzidine (Bio-Rad, USA) and hydrogen peroxide were added, and the reaction was stopped by adding 50 μ l of 1 N sulfuric acid. The intensity of the colorimetric signal was measured using a Thermo Fisher plate reader at 450 nm.

Multi-cytokine test

IP-10, MIG, IL-1, IL-4, MIP1a, IL-12, IL-13, TNF- α , and IL-10 were quantified in QuantiFERON supernatant using a microsphere-based assay (ProcartaPlex™ Human, Thermo Fisher Scientific, USA) and a Magpix apparatus (Merck Millipore, USA) following the manufacturer's instructions. Cytokine levels were obtained by subtraction of the value in the unstimulated tube (Nil) from the value obtained following stimulation with TB antigens.

Single cytokine enzyme-linked immunosorbent assay

The most discriminant cytokines between confirmed TB and control groups were quantified in QuantiFERON supernatants using ELISA kits because this method is more accessible for cytokine level measurements. IL-2 was quantified by DuoSet ELISA Development Kits (Bio-Techne, USA) and MIG by Standard ELISA Development Kits (PeproTech, USA). QuantiFERON supernatants were diluted in a 1:2 ratio for all cytokines. Cytokine levels were obtained by subtraction of the value in the unstimulated supernatant from the value obtained following peptide stimulation. Results were expressed in pg/ml.

Statistical analyses

The recruitment of 100 children with confirmed TB allowed estimating a target sensitivity of 85% of the diagnostic score with a precision of $\pm 7\%$. To enroll 100 children with confirmed TB, we estimated that we should enroll approximately 500 children with presumptive TB. To reach a precision of $\pm 6\%$ around a specificity of 85%, 150 controls had to be enrolled.

A random forest classification analysis of biomarker values was used to identify the most discriminant cytokines between confirmed TB and control groups. The random forest method grows a collection of decision trees constructed using different bootstrap samples of the data. Each node is split using the best among a subset of predictors randomly chosen at that node. We used the mean decrease in Gini index to define the most discriminant biological factors. The selected markers were then analyzed by ELISA, limited to a maximum of two or three markers, to evaluate the performance of the TB score and ensure that the algorithm could be feasibly implemented in the field.

We dichotomized quantitative markers, including the IL-2/IFN- γ ratio, according to the best cutoff derived from receiver operating characteristic (ROC) curves using the Youden index. The IL-2/IFN- γ ratio was categorized into three groups: below the threshold, equal or over the threshold, and QuantiFERON-negative.

To construct the diagnostic score, a logistic regression analysis was done for all categorical biological factors selected in the previous steps. To gauge their independent association with TB, a variable was selected and entered into a multivariable logistic regression model if its *P*-value was lower than 0.20 in the univariate analysis. A backward procedure was used to select the final model, in which a numerical value was assigned to each of the significant variables, in relation to their β parameter (logistic regression estimates). The discriminatory capacity of the score was determined by calculating the area under the curve. To facilitate the interpretation of the algorithm, the best discriminant cutoff was brought up to 100 with a multiplier factor. This same multiplier factor was applied to each β parameter, which were then rounded to the nearest integer, and then to the nearest appropriate value for simplicity and ease of use. We used the bootstrap method to internally validate the score by sampling with replacement for 1000 iterations. Statistical analyses were conducted with SAS Enterprise Guide version 9.1 (SAS Institute, Cary, NC, USA) and R version 3.3.1 (R Foundation for Statistical Computing, Vienna, Austria).

Results

A total of 790 children were recruited between December 2013 and December 2015 from the two study sites, including 628 children suspected of having TB and 162 controls. Among the 628 children screened, 591 had presumptive TB based on the presence of at least two of the WHO's screening symptoms, of which 105 had a TB diagnosis confirmed by culture and/or Xpert MTB/RIF assays (Figure 1).

Cytokine assessment from QuantiFERON supernatants and Ag85B antibody testing were available for 107 children, including 37 from the confirmed TB group. The characteristics of children with indeterminate results in the confirmed TB group (*N* = 68) and in the control group (*N* = 81) are presented in supplemental Tables S1 and S2, showing similar patterns, with slight differences in *z* scores in the control group. The characteristics of children included in the study are presented in Table 1. A total of 24 children (64.9%) had a positive direct smear examination, 32 (86.5%) had a positive culture, and 32 (86.5%) had a positive Xpert TB test. Among children in the TB group, QuantiFERON results were positive for 32 (86.5%), whereas 16 (22.9%) children in the control group showed positive results (*P* < 0.0001).

The TB group and control group had a similar sex ratio and age distribution. Fifteen children (40.5%) in the TB group had a history of TB exposure in the last 2 years or have already been treated for TB. Child growth was impaired in the confirmed TB group, while it was normal in the control group. In the TB group, 97.3% (*n* = 36) of children had abnormal chest radiography results suggestive of TB (data not shown). Nineteen children (51.3%) were infected with HIV in the TB group, and 31 (44.3%) in the control group. The median hemoglobin rate was lower in the confirmed TB group than in the control group (8.60 g/dl [interquartile range 7.40-9.60] vs 12.30 g/dl [interquartile range 11.30-13.30], respectively; *P* < 0.01).

The random forest classification analysis identified Ag85B, IL-2/IFN- γ ratio, MIG, and IP-10 as the biomarkers most strongly associated with TB (Sup Figure 1). We then tested IL-2, IFN- γ , MIG, and IP-10 by ELISA to determine the scoring system. IP-10 results were eventually not considered for the final algorithm to stay within four biomarkers because MIG and IP-10 responses were strongly correlated (Sup Figure 2).

Lower values of IL-2/IFN- γ ratio and higher levels of Ag85B antibodies and MIG were observed in the confirmed TB group compared with controls (Sup Figure 3). Ag85B antibodies, MIG level, and IL-2/IFN- γ ratio were independently associated with active TB in the multivariable regression analysis (Table 2). Using ROC curves and the Youden index, we established the respective thresholds

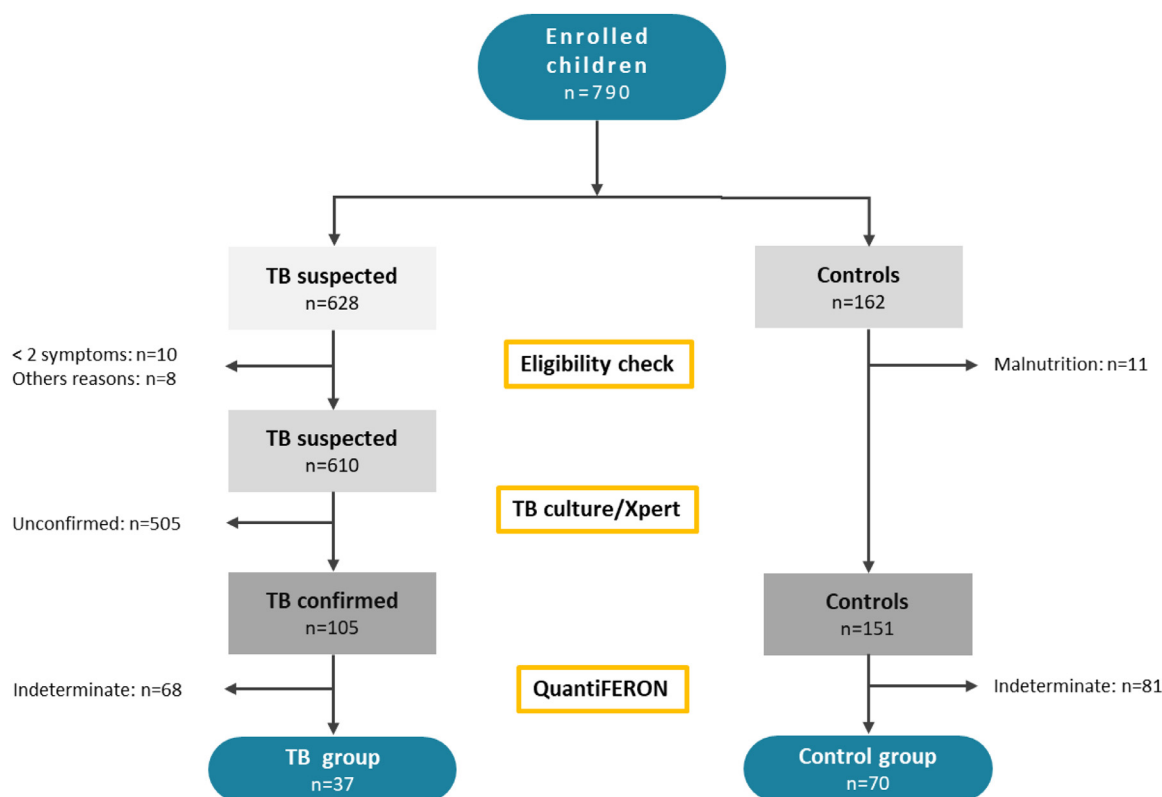


Figure 1. Flowchart. TB, tuberculosis.

of MIG, Ag85B antibodies, and IL-2/IFN- γ ratio to better discriminate children with confirmed TB from control children (data not shown). Neither age nor HIV infection changed the association between each marker and TB (i.e., absence of interaction in the models; data not shown). Because one of the IL-2/IFN- γ classes was not contributive, this variable was dichotomized (IL-2/IFN- γ index <48.6 vs IFN- γ negative or IL-2/IFN- γ >48.6).

The score derived from these markers using the logistic regression estimates is presented in Table 3. The ROC curve derived from our score showed an area under the curve of 0.95 (0.91-0.99), providing 91% sensitivity and 88% specificity (Sup Figure 4). The internal bootstrap validation provided the following 95% confidence intervals (CIs) for the performance of our scoring system: sensitivity 71-97% and specificity 79-99%. The overall concordance rate between the TB diagnostic score and microbiological confirmation assays was 0.878 (95% CI 0.817-0.940) and 0.804 (95% CI 0.728-0.879) between the QuantiFERON and microbiological confirmation assays (Figure 2).

Discussion

We evaluated the performance of a blood test for TB diagnosis based on a combination of serological and T-cell cytokine release assays in children. The findings from this pilot study demonstrated that the diagnostic algorithm met the accuracy criteria specified in the WHO's target product profiles for a non-sputum-based test in children [3]. The results obtained in this initial phase suggest that our TB diagnostic score holds potential applicability in high HIV and TB burden settings.

Development of a TB diagnosis test based on a combination of host biomarkers in blood is considered a priority [15]. A sensitivity of 59.8% (95% CI 50.8-68.4) and a specificity of 90.3% (95% CI 85.5-94.0) have been recently reported in children with con-

firmed TB using the Cepheid *M. tuberculosis* host response prototype cartridge, which explores host gene transcriptional signatures in blood [16]. Numerous studies suggest that testing antibodies and cytokines produced in response to TB antigens can be beneficial in TB diagnosis; however, their diagnostic performance may not be sufficient when considered individually [15]. In this study, we hypothesized that a combination of biomarkers of the immune response has the potential to add value to the QuantiFERON assay.

The IFN- γ results from the QuantiFERON assay constitute the first marker included in the scoring system. This commercial assay has undergone extensive assessment over the past two decades. While the test performs well for diagnosing TB infection in children [7,17], it still struggles to differentiate between active and latent TB infection, particularly in children with advanced HIV infection [18]. We did not observe changes in the association between each marker and TB in our study according to HIV status, but studies have shown that the results of IGRA tests in children with HIV depend on their immunological state [11].

Both MIG and IL-2, two cytokines we quantified in the QuantiFERON supernatant, contributed to establishing our diagnostic algorithm along with IFN- γ . IL-2 secretion has been extensively investigated as an alternative or complementary marker to IFN- γ , as well as IP-10 [19]. We observed that the MTB-specific T-cell signature associated with active TB in children was characterized by a preferential IFN- γ production relative to IL-2 and a stronger secretion of IP-10 and MIG. Our findings align with previous studies that have assessed the cytokine signatures associated with active TB in adults and children. A relative dominance of IFN- γ secretion compared with IL-2 has been observed in adults during active TB compared with latent TB [20-22]. Reduced IL-2 secretion relative to IFN- γ production may be linked to a defect in host control of MTB, contributing to the development of TB disease [23]. Hence, the IL-2/IFN- γ ratio has the potential to serve as a diag-

Table 1
Characteristics of children in confirmed TB and control groups.

		Confirmed TB group (n = 37)	Control group (n = 70)	P-value
Sex of the child	Female	19 (51.4%)	30 (42.9%)	0.42
	Male	18 (48.6%)	40 (57.1%)	
Age in categories	<5 yrs	15 (40.5%)	16 (22.9%)	0.76
	≥5 yrs	22 (59.5%)	54 (77.1%)	0.80
Age (years)		8.9 (2.3-12.0)	9.5 (5.6-14.0)	0.63
History/previous treatment for TB	Not known	0	1 (1.4%)	<0.0001
	Negative	22 (59.5%)	69 (98.6%)	
	Positive	15 (40.5%)	0	
Anthropometric data				
Weight for age Z-score (age <5 years)		-3.72 (-4.10; -3.16) ^a	-0.66 (-0.95; -0.03)	<0.0001
Weight for age Z-score (age [5-10 years])		-1.10 (-4.67; 0.49) ^a	-0.27 (-1.00; 0.69)	0.05
Height for age Z-score (age <5 years)		-2.46 (-3.82; -2.15) ^b	-1.58 (-2.35; -0.74) ^c	0.02
Height for age Z-score (age ≥5 years)		-1.44 (-2.20; -0.19) ^c	-1.26 (-2.19; -0.27)	0.40
Weight for height Z-score (age <5 years)		-2.96 (-3.58; -1.67) ^b	0.84 (-0.80; 1.33) ^c	<0.0001
Body mass index Z-score (age ≥5 years)		-2.40 (-3.71; -1.13) ^d	0.25 (-0.68; 0.89)	<0.0001
Malnutrition severity ^e	No malnutrition or mild	3 (8.1%)	21 (30.0%)	0.010
	Moderate to severe	34 (91.9%)	49 (70.0%)	
Laboratory results				
MTB direct examination (+)		24 (64.9%)	-	
MTB culture (+)		32 (86.5%)	-	
Xpert (+)		32 (86.5%)	-	
QuantiFERON (+)		32 (86.5%)	16 (22.9%)	<0.0001
HIV status: polymerase chain reaction + rapid test	HIV negative	18 (48.7%)	39 (55.7%)	0.54
	HIV positive	19 (51.3%)	31 (44.3%)	
CD4 count (absolute count)		192 (130-558) ^f	-	-
CD4 count (percentage)		13.8 (9.6-15.0) ^f	-	-
Hemoglobin		8.6 (7.4-9.6) ^d	12.3 (11.3-13.3)	0.017

Data are presented as median (interquartile range) or n (%). MTB, *Mycobacterium tuberculosis*; TB, tuberculosis.

^a Six missing data.

^b Three missing data.

^c One missing data.

^d Five missing data.

^e Guideline: Assessing and Managing Children at Primary Health-Care Facilities to Prevent Overweight and Obesity in the Context of the Double Burden of Malnutrition: Updates for the Integrated Management of Childhood Illness (IMCI). Geneva: World Health Organization; 2017. Table 1, World Health Organization (WHO) classification of nutritional status of infants and children. Available from: <https://www.ncbi.nlm.nih.gov/books/NBK487900/table/fm.s1.t1/>.

^f 37 missing data.^gFour missing data.^hEight missing data.ⁱ15 missing data.^j19 missing data.^k39 missing data.

Table 2
Significant biological factors in logistic regression analysis to predict the risk of tuberculosis.

Biological factors	Univariate analysis	Multivariable analysis	
	Crude OR (95% CI)	Adjusted odds ratio (95% CI)	P-value
Antigen 85B antibodies ≥0.76 vs <0.76	3.32 (1.44-7.65)	9.87 (1.94-50.32)	0.006
MIG ELISA ≥190 vs <190	13.64 (5.21-35.72)	8.36 (2.25-31.02)	0.002
IL-2 ELISA/IFN-γ ratio <48.6 vs IFN-γ negative or IL-2 ELISA/IFN-γ ratio ≥48.6	44.55 (12.85-154.41)	58.23 (10.34-327.99)	<0.001

CI, confidence interval; ELISA, enzyme-linked immunosorbent assay; OR, odds ratio.

nostic biomarker for distinguishing active from latent TB infection [24–27]. In a setting with low TB incidence, we recently identified a similar threshold for the IL-2/IFN-γ ratio in 195 adults with active vs latent TB infection (≤ 47.0 vs ≤ 48.6) [20].

The fourth marker included in our scoring system relates to the systemic antibody response against the Ag85 complex, which consists of three secreted proteins crucial for MTB attachment and macrophage invasion. Among the MTB secretory proteins, the Ag85 complex members are among the most abundant and immunogenic proteins secreted by *M. tuberculosis* [28]. Previous studies have reported interesting results for TB diagnosis by testing antibodies directed against Ag85 in children [5,28–30] and adults [29,31,32]. The WHO recommends using new treatment decision algorithms (TDAs) that include a molecular test for diagnosing and managing TB in children [33]. The TB score should be further evaluated in a diagnostic accuracy cohort study against both micro-

biological and composite reference standards, and subsequently within the TDA, either as a triage test or as an additional test for children who test negative for MTB DNA but exhibit clinical and radiological signs consistent with TB. Other tests could be included, particularly those based on the stimulation of lymphocytes by the heparin-binding hemagglutinin adhesion protein, which have shown a promising ability to distinguish between active and latent forms of TB [34].

Although our bi-centric study recruited a large number of confirmed TB cases and encompassed the spectrum of children with suspected TB in a high HIV prevalence area, our study has several limitations. The main limitation was the unavailability of QuantiFERON results for many children. The QuantiFERON assay, which uses whole blood in tubes containing antigenic peptides, is a simple approach to studying T-cell response to MTB. However, time constraints for transporting and processing the specimen, and the

Table 3
Score of the significant biological factors according to the logistic regression estimates (β parameter).

Biological factors	β estimate from logistic regression	Score ^a	Rounded score
Ag85B Abs <0.76	0	0	0
Ag85B Abs \geq 0.76	2.289458	72.07	72
MIG <190	0	0	0
MIG \geq 190	2.122897	66.83	67
IFN- γ negative or IL-2 ELISA/INF- γ ratio \geq 48.6	0	0	0
IL-2 ELISA/INF- γ ratio <48.6	4.064508	127.95	128

Ag85B Abs, antigen 85B antibodies; ELISA, enzyme-linked immunosorbent assay.

^a Score = β estimate \times multiplier factor (with 31.48 as multiplier factor).

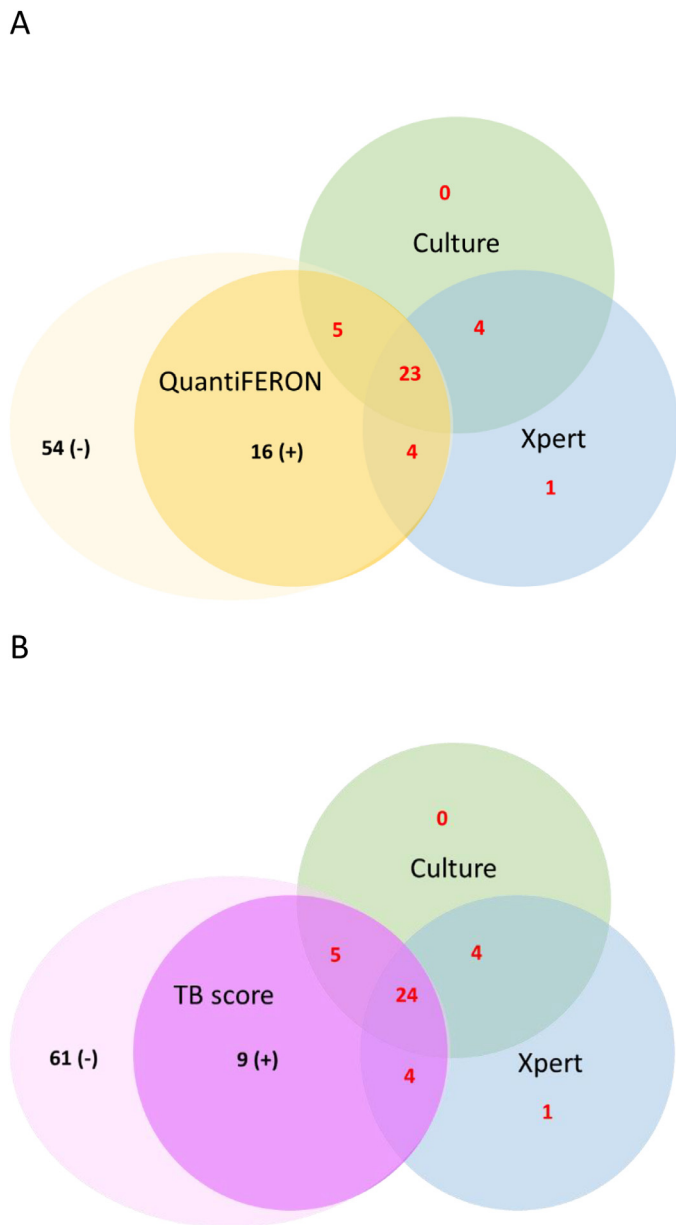


Figure 2. Venn diagram depicting laboratory results. Distribution of the results among TB confirmed children (red numbers) and controls (black number): Mycobacterium tuberculosis culture, Xpert PCR, and QuantiFERON assay (A); Mycobacterium tuberculosis culture, Xpert PCR, and TB diagnostic score (B). PCR, polymerase chain reaction; TB, tuberculosis.

time required to obtain results are some of the limitations of diagnostic assays based on T-cell activation. In our study, we encountered difficulties in incubating the QuantiFERON tubes within the 16 hours recommended by the manufacturer. Consequently, considering their results as indeterminate, we were forced to exclude a large number of participants. These difficulties were mainly due to high turnaround of both health care workers employed part-time for this research and laboratory staff. In addition, the laboratory centrifuge was not operational for several months, without immediate replacement solutions. The high proportion of invalid QuantiFERON tests was discovered at the end of the study, when cytokines were planned to be analyzed, which prevented corrective actions. IGRA have been associated in some studies with higher rates of indeterminate results in children compared with adults, but other studies have reported a low proportion of indeterminate results in children [35]. The utmost consideration must be given to the pre-analytic steps that can be challenging in the field. Fully automated assays [36] and simplified versions of IGRA with potential for point-of-care use may facilitate the implementation of IGRAs in the field [37]. These missing data reduced the precision of the diagnostic performance, resulting in wide 95% CIs, but it was unlikely to have introduced any selection bias given that the children with and without missing data had comparable characteristics (Tables S1 and S2). Another limitation is the absence of matching for age and HIV status between TB-confirmed participants and controls, although the characteristics between the two groups were similar.

Besides these performances against Xpert MTB and/or MTB culture as gold standards, the potential of this algorithm should be further evaluated in a diagnostic cohort, including children with clinical and radiological features suggestive of TB or clinically indeterminate for TB, and according to their response to treatment during follow-up. In our study, while the scoring system required four ELISA tests, these markers could potentially be assessed simultaneously using a multiplex method. Considering the 16- to 24-hour incubation time for the QuantiFERON assay, it does not meet the WHO's requirement for a rapid test based on non-sputum specimens, but the results of our algorithm can be obtained within 1.5 days after admission. However, this is an issue in low- and middle-income countries and could lead to loss to follow-up for some children.

The diagnostic gaps for childhood TB remain considerable in settings with high TB incidence and resource constraints. Our findings suggest that testing both serological and T-cell markers against MTB, alongside the IGRA, improves TB triage and diagnosis in childhood, regardless of HIV status and age. The implementation feasibility and performance of the TB score, with a sensitivity and specificity >85%, require further validation in the target population of all children presenting with suspected TB.

Declarations of competing interest

The authors have no competing interests to declare.

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Ethical approval

The study was approved by the national ethical committee for medical research in Zambia, and recorded in ClinicalTrials.gov (NCT02573623).

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Author contributions

ET: Study design, supervision, manuscript writing, and editing; MM, MWT, DMC: Data collection, patient recruitment, laboratory investigations, clinical data analysis, and contributions to analysis interpretation; KB: Laboratory investigations, data analysis, manuscript writing, and editing; AP, PAR: Laboratory investigations, data analysis; MR, LK: Methodology, laboratory support; MP, RV, SED, MD: Data management, statistical analysis, and analysis interpretation; CK: Field coordination and supervision; JPM: Laboratory supervision, manuscript writing, data interpretation, and review; NN, PVP: Project supervision, conceptualization, manuscript writing, and data interpretation. All authors contributed to the manuscript preparation and gave final approval for submission.

Supplementary materials

Supplementary material associated with this article can be found, in the online version, at [doi:10.1016/j.ijid.2024.107248](https://doi.org/10.1016/j.ijid.2024.107248).

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