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TITLE:

**ADVANCING THE DIAGNOSIS OF PEDIATRIC
TUBERCULOSIS: FROM IMMUNOLOGICAL TO
METABOLOMIC TOOLS**

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CHAPTER 1

Pediatric Tuberculosis: Clinical Features and Diagnostic Challenges

1. Global burden and pediatric impact

Tuberculosis (TB) remains one of the most significant infectious diseases affecting people worldwide. Despite being a preventable and curable disease, TB continues to impose a heavy global burden with 10.8 million new cases and 1.25 million deaths estimated in 2023. Among them, about 1 million children aged below 15 years developed TB disease, accounting for 12% of the global burden and making TB a major cause of childhood mortality and a research priority for the World Health Organization (WHO) (1).

In low-incidence countries such as Italy (4.9/100,000 newly reported cases in 2023), certain groups are considered at higher risk of TB: migrants and travelers to endemic regions (Eastern Europe, Africa, Asia, Latin America), internationally adopted children, institutionalized individuals, people living in overcrowded housing conditions and immunocompromised individuals (2).

2. Pathogen, transmission and natural history

The etiologic agent of TB is the *Mycobacterium tuberculosis* complex, a group of closely related mycobacteria that includes *M. tuberculosis* (*Mtb*) (by far the most frequent cause of human infection), *M. bovis*, *M. africanum*, and other less common mycobacteria. Transmission of *Mtb* occurs via airborne droplets expelled by an infectious individual. The likelihood of infection depends on several factors, including the infectiousness of the source case, duration and intensity of exposure, environmental conditions, and host susceptibility (3).

In children, infection is most often acquired from an adult household contact, as pediatric cases are generally paucibacillary and therefore rarely contagious (4).

However, not all individuals infected with *Mtb* progress to active disease.

After exposure to an infectious tuberculosis case, three main conditions may arise, reflecting the different stages of infection and disease development (4):

- **Contact:** a person with a history of recent contact (<3 months) with a contagious TB case, without evidence of infection, and with normal clinical and radiological findings.
- **Latent TB infection (LTBI):** infection with *Mtb* (demonstrated by a specific immune response to *Mtb*-derived protein antigens), in the absence of clinical or radiological evidence of disease.
- **TB disease:** a clinical condition characterized by *Mtb* infection with clinical and/or radiological evidence of disease.

In recent years, the World Health Organization (WHO) has adopted the term tuberculosis infection (TBI) in place of LTBI to emphasize the continuous spectrum between infection and disease. However, to avoid ambiguity within the context of this thesis, the term LTBI will continue to be used hereafter.

Risk factors for progression from infection to disease include patient's age, malnutrition, chronic illnesses, congenital or acquired immunodeficiency (e.g., HIV infection, immunosuppressive treatment), and recent infection (<2 years) (5).

Children represent a particularly vulnerable group in the global TB burden. Overall, about 5–10% of individuals infected with *Mtb* develop active TB disease during their lifetime, most commonly within the first five years after initial infection (5,6). The risk of progression to disease is markedly higher in young children—reaching approximately 50% in infants under two years of age—and declines with age, approaching adult levels by school age. Moreover, disease progression in children tends to occur more rapidly, in approximately 90% of cases within the first 12 months following infection (7,8). Infants and young children—particularly those under two years—are also at increased risk of severe and disseminated forms of TB, such as miliary disease and tuberculous meningitis (TBM), both of which are associated with high morbidity and mortality (7).

Despite its substantial impact, pediatric TB has historically received less attention than adult disease, and the diagnostic challenges specific to children have further contributed to its underdiagnosis, underreporting, and delayed treatment (9).

3. Diagnostic challenges in children

3.1 Immunological tests: TST and IGRA

Immunologic diagnostic tests, such as the tuberculin skin test (TST) and interferon- γ release assays (IGRAs), can identify TB infection but cannot discriminate LTBI from active disease, as both detect the cell-mediated immune response that develops following infection with *Mtb* (10).

TST consists of the intradermal injection of tuberculin purified protein derivative (PPD). Previous exposure results in a local delayed-type hypersensitivity reaction within 24–72 hours. IGRA is an in vitro blood test that measures interferon-gamma production by T-cells in response to *Mtb* antigens. These antigens are absent from BCG vaccines and from most non-tuberculous mycobacteria (10). It is important to note that IGRA use in children, especially those under two years of age, remains poorly standardized (11).

A negative result on immunological tests does not definitively rule out TB infection. With a sensitivity of approximately 80–90%, these tests may yield false-negative results, particularly in individuals with recent exposure or immunocompromising conditions (12).

3.2. Clinical, radiological and microbiological evaluation

Radiological, clinical, and microbiological findings are often insufficient to clearly differentiate LTBI from active TB in children. Clinical manifestations vary considerably with age. In young children, symptoms are often subtle and nonspecific, with up to 50% of cases remaining asymptomatic. In contrast, disease presentation in adolescents increasingly resembles that observed in adults (13,14).

Radiological features of pediatric TB are heterogeneous and age-dependent. In young children with pulmonary disease, the most common chest X-ray finding is enlarged perihilar and/or paratracheal lymph nodes, often associated with limited parenchymal involvement (13). Other patterns include atelectasis, pleural effusion, miliary nodules, or cavitation (13).

Sputum-based microbiological diagnostics, the cornerstone of adult TB diagnosis, are often unfeasible in children due to challenges in specimen collection and low bacterial

loads (4). Alternative samples, such as gastric aspirates or induced sputum, increase diagnostic yield in children but remain limited in sensitivity (4,15).

Evaluation of a child with suspected TB is complex and should integrate immunological, clinical, radiological, and microbiological findings, as well as epidemiological history and social risk factors (9).

4. Therapeutic management

Therapeutic management differs between LTBI and active TB.

The standard treatment for drug-susceptible pulmonary TB involves four first-line drugs: rifampicin (R), isoniazid (H), pyrazinamide (Z), and ethambutol (E).

TB treatment includes a 2-month intensive phase followed by a continuation phase of 2–4 months. In the intensive phase, TB bacilli are rapidly killed to prevent disease progression, transmission and development of drug resistance. In the continuation phase, dormant bacilli are eliminated to achieve cure and prevent relapse (10).

The intensive phase consists of a four-drug regimen (HRZE) or a three-drug regimen (HRZ). The three-drug regimen (excluding ethambutol) may be used in children with uncomplicated pulmonary TB or tuberculous lymphadenitis living in settings with low HIV prevalence and low isoniazid resistance (10).

Regardless of the intensive-phase regimen, the continuation phase consists of a two-drug regimen (isoniazid and rifampicin), historically extended for an additional four months, resulting in a total treatment duration of six months for pulmonary TB (10).

In children and adolescents aged 3 months to 16 years with non-severe TB, a 4-month treatment regimen is now recommended according to the most recent WHO guidelines, marking a significant shift from the traditional 6-month course (10).

Non-severe TB is defined as peripheral lymphadenitis, intrathoracic lymphadenopathy without airway compression, uncomplicated pleural effusion, or non-miliary, non-cavitary pulmonary disease involving a single lobe (10).

Extrapulmonary TB generally requires longer therapy depending on the site and severity of disease (10).

During treatment, careful monitoring of therapeutic response and adherence is essential to ensure cure, prevent the emergence of drug resistance, and promptly identify potential adverse effects. The main adverse events include hepatotoxicity (associated with isoniazid, rifampicin, and pyrazinamide), peripheral neuropathy (associated with isoniazid), hyperuricemia and arthralgia (associated with pyrazinamide), and optic neuritis (associated with ethambutol) (10).

Treatment of LTBI substantially decreases the risk of progression to active disease, with an estimated risk reduction of about 60% (16). Historically, the only approved regimen consisted of 6–9 months of isoniazid monotherapy. In recent years, several alternative regimens have been tested in comparison to isoniazid monotherapy (taken as golden standard), approved by international authorities and progressively introduced in clinical practice based on exposure and availability of single drugs. Those regimens include: isoniazid plus rifapentine weekly for 3 months, isoniazid plus rifampicin for 3 months, or rifampicin alone for 4 months (10).

5. Rationale and aims of the project

The inability to reliably differentiate LTBI from active TB disease represents a major diagnostic dilemma and a central obstacle in pediatric TB management. Misclassification of these states has significant clinical consequences: underdiagnosis of active TB can delay lifesaving treatment, while overdiagnosis leads to unnecessary exposure to prolonged multidrug regimens. Addressing this gap is essential for both optimizing individual patient care and improving the efficiency of TB prevention programs. These limitations underscore an urgent unmet need: diagnostic tools that are both sensitive and specific in children, based on easily obtainable specimens, and capable of informing clinical decisions early in the disease course. Ultimately, pediatric TB cannot be viewed as a peripheral issue within the global TB epidemic. It is both an indicator of transmission dynamics and a determinant of future disease burden. Addressing the diagnostic and clinical gaps in children will not only reduce morbidity and mortality in this vulnerable population but also contribute to breaking the cycle of transmission and advancing global TB elimination. The present project was carried out on a pediatric population living in a low TB incidence

setting. Understanding the local epidemiology of TB in low-incidence countries is crucial to accurately estimate the risk of transmission, raise disease awareness, and maintain a high index of suspicion among healthcare professionals. Moreover, local epidemiological patterns directly influence the performance of diagnostic tests, as their accuracy—particularly in terms of sensitivity, specificity, and predictive values—varies according to disease prevalence.

Hence, this project addresses the epidemiology of TB in a low-prevalence setting and primarily focuses on diagnostic challenges, with particular attention to the evaluation of currently available diagnostic tests and the validation of novel tools aimed at improving the differential diagnosis between LTBI and active disease.

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CHAPTER 2

Epidemiology and Clinical Features of Pediatric TB: Insights from a Regional Reference Center in Southern Italy

Tuberculosis poses its highest burden in low- and middle-income countries, yet it remains a relevant public health challenge in high-income settings as well (1).

Prior to the COVID-19 pandemic the WHO European Region, which includes both high- and low-incidence countries (such as European Union/European Economic Area, EU/EEA), was showing a consistent, though slow, decline in TB incidence and mortality, reflecting improved case detection, access to treatment, and implementation of preventive measures (2). However, the onset of the COVID-19 pandemic in 2020 caused a substantial disruption in TB control activities, leading to a marked decline in case notifications rather than a true reduction in disease burden (3).

In 2020 and 2021, TB notifications in the European Region dropped sharply — by around 25% compared with 2019 — due to reduced healthcare access, diagnostic delays, and overwhelmed health systems. During this period, estimated TB incidence remained relatively stable (approximately 230,000 cases per year, or 24 per 100,000 population), suggesting that many cases went undiagnosed or unreported (4).

In 2022 and 2023, the number of TB notifications increased again, approaching pre-pandemic levels. The ECDC report indicates a 10% rise in reported TB cases between 2021 and 2023, consistent with a rebound in detection rather than an epidemiological surge (5,6). Nevertheless, the temporary setback caused by COVID-19 has delayed progress toward the End TB milestones, with the decline in incidence from 2015 to 2023 reaching only 27%, far below the 50% reduction target set for 2025 (1,7).

The epidemiological pattern of TB in Italy has mirrored that of the broader European Region. In 2023, a notification rate of approximately 4.9 per 100,000 inhabitants, confirmed its status as low-incidence country (6).

However, national trends may overlook important regional variations in TB epidemiology. Understanding and studying one's specific local setting is therefore

essential to accurately assess disease dynamics, identify high-risk populations, and implement effective, context-adapted public health interventions.

Within the research group of the Pediatric Infectious Diseases Unit at the University of Naples “Federico II” — the sole regional referral center for pediatric TB in Campania Region — I contributed to the collection and analysis of a uniquely comprehensive dataset that closely mirrors the true regional epidemiology.

Before COVID-19 pandemic, our findings over the ten-year period 2009–2018 showed a progressive increase in pediatric TB notification rates, rising from 0.44 to 1.84 per 100,000 inhabitants under 18 years. This increase was strongly correlated with migration flows ($R^2 = 0.93$, $P < 0.0001$), highlighting the influence of demographic shifts on disease dynamics. Importantly, children of foreign origin were disproportionately affected, exhibiting poorer clinical outcomes and accounting for all fatal cases and losses to follow-up.

These findings highlight the vulnerability of migrant families, linked to socioeconomic disadvantage, language barriers, limited healthcare access, and reduced awareness of TB risk, underscoring the need for tailored management strategies to ensure early detection, treatment completion, and equitable clinical outcomes in this high-risk population.

We reported these findings in the study *“Increasing Tuberculosis Rates and Association with Migration in Children Living in Campania Region, Southern Italy: A 10-Year Cohort Study,”* reproduced below – Annex 1(8).

The COVID-19 outbreak dramatically altered this scenario. In our center, we observed a 73% reduction in pediatric TB notification rates in 2021 compared to 2019 (0.38/100,000 vs 1.46/100,000). However, this apparent decline was accompanied by a marked increase in disease severity at presentation, with 41.6% of cases classified as severe during 2020–2021, compared to 15.7% in the pre-pandemic decade.

We speculated that this reduction did not reflect a true decrease in *Mtb* transmission, but rather underdiagnosis and underreporting resulting from restricted healthcare access, which led to delayed presentations and worsened clinical outcomes, as many children reached medical services only when urgent intervention was required.

We suggested that the suspension or downsizing of TB diagnostic and surveillance services, the reallocation of healthcare resources, and limited patient access to care during lockdowns played a decisive role in this phenomenon.

In our setting, we highlighted that the restoration of standard TB services alone was insufficient to reverse the trend, as notification rates remained lower even after the lifting of restrictions. This raised concern that a substantial number of undetected pediatric TB cases might persist in the community, potentially fueling future transmission and worsening long-term outcomes.

We therefore suggested that targeted public health measures—including active case finding, strengthened TB surveillance, and improved access for vulnerable populations—were needed to mitigate the long-term impact of this diagnostic gap and prevent a post-pandemic resurgence of pediatric TB.

These findings were reported in our study *“Effect of the COVID-19 Pandemic on Pediatric Tuberculosis: Decrease in Notification Rate and Increase in Clinical Severity,”* reproduced below – Annex 2 (9). Subsequently, European surveillance data confirmed our observations on a broader scale (3,4,5,6).

In children, who are more vulnerable to rapid disease progression, the previously described dynamics are particularly concerning (10). Furthermore, children represent a sentinel group in TB epidemiology, as pediatric cases are strongly indicative of ongoing transmission within communities and often highlight gaps in surveillance and prevention (11). Our experience shows how a combination of migration-driven epidemiological shifts and pandemic-related diagnostic delays can converge to exacerbate TB severity in a low-incidence context. Furthermore, they underline the importance of maintaining robust pediatric TB surveillance systems to detect changes in incidence and severity, ensuring adequate care for vulnerable populations.

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ORIGINAL STUDIES

Increasing Tuberculosis Rates and Association With Migration in Children Living in Campania Region, Southern Italy: A 10-Year Cohort Study

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Background: Italy is classified as a low tuberculosis (TB) incidence country (rate 6.5/100,000 inhabitants). However, the Campania Region Pediatric Reference Centre (CRRC) observed an increase in TB, contemporarily with a rise in migration. Our aim was to investigate trends in TB notification rates, association with migration, and changes in clinical outcomes of children living in Campania.

Methods: We conducted a prospective cohort study (January 1, 2009–December 31, 2018), including children <18 years who received diagnosis of TB at the CRRC. Yearly crude TB incidence rates and relative confidence interval (95% CI) were calculated. Two main outcome measures were considered: loss to follow-up and poor clinical outcome, including prolonged or second-line treatment, sequelae, or death.

Results: Overall 146 children (52.1% male; median age, 50 months; interquartile range, 96.33) received a diagnosis of TB. TB incidence rates increased from 0.44 cases (95% CI: 0.16–0.97) per 100,000 inhabitants <18 years of age in 2009 to 1.84 cases (95% CI: 1.15–2.79) in 2018 ($P < 0.05$) and linearly correlated with the rate of migrants ($R^2 = 0.9272$; $P < 0.0001$). Ziehl-Neelsen-positive children had an increased likelihood of poor clinical outcomes (odds ratio, 4.83; 95% CI: 1.28–18.2; $P = 0.020$). Compared with Italians, foreign children showed a lower likelihood of cure without sequelae (49.3% versus 67.9%; $P < 0.001$; odds ratio, 0.45; 95% CI: 0.23–0.89; $P = 0.02$). They accounted for all fatal cases and loss to follow-up.

Conclusion: Pediatric TB rate in Campania increased in the last 10 years in association with the increase in migration. Emphasizing national TB rates may disregard important differences in local infection trends and limit medical awareness about TB. Foreign children may need tailored management programs.

(*Pediatr Infect Dis J* 2020;39:1017–1022)

INTRODUCTION

Tuberculosis (TB) remains a public health concern in the European Union/European Economic Area (EU/EEA), although the overall notification rate is approaching the low incidence threshold with 10.7 cases per 100,000 inhabitants.^{1,2} One-third of all TB cases reported were of non-European origin. This proportion increased

from 27% in 2013 to 33% in 2017. About 2500 cases were notified in children younger than 15 years living in the EU/EEA.

Italy is classified as a low incidence country with about 4000 TB cases notified in 2017 and a notification rate of 6.5 cases per 100,000. In line with European data, children accounted for 4.3% of cases, with a rate of 2.1 per 100,000.³

In recent years, the EU/EEA countries have witnessed an unprecedented volume of migration, with more than 1 million migrants arriving in Europe yearly. Together with Greece, Italy is welcoming the largest number of migrants, especially those reaching Europe through the Mediterranean routes.³ According to the Italian Institute of Statistics (ISTAT), more than 5 million foreigners are residing in Italy, with an increasing number of children and unaccompanied foreign minors.⁴ In 2017, Campania hosted 16,677 new refugees, accounting for about 10% of all migrants reaching the national territory.⁵

During the last 10 years of activity, an increase in the number of TB cases was observed in the regional reference center for pediatric TB, which led to the creation of a Pediatric Register to record and process data prospectively.

The aim of the present study was to calculate the annual TB rates and evaluate the trend of incidence over a 10-year observation period. Parallely, we reviewed data about migration in Campania Region in the same period and estimated its impact on the local TB epidemiology and clinical outcomes.

METHODS

We used a prospective Register of Pediatric TB to conduct a single-center cohort study in children seen at the Pediatric Infectious Diseases Unit (PIDU) of the University of Naples Federico II between January 1, 2009, and December 31, 2018. This is the only reference center for the management of children with TB in Campania, the region with the highest population density in Italy with more than 5 million inhabitants and about 1 million children.

Definitions and Enrollment

All subjects 18 years of age or younger who received a diagnosis of active TB at the PIDU were included in the study.

Diagnosis of TB was based on an immune response to stimulation by *Mycobacterium tuberculosis* antigens (positive skin test and interferon-gamma release assays), with clinical or radiologic evidence of TB. Children were classified as having pulmonary or extrapulmonary TB, according to the European Centre for Diseases Control and Prevention criteria.² Those with multiple disease localizations were classified taking into account the most severe clinical manifestation (eg, a child with pulmonary TB and meningitis was classified as TB meningitis). Children exposed to cases of TB or receiving a diagnosis of latent TB infection (LTBI) were excluded.

Data Collection and Outcome Measures

A standardized database was used to collect demographic data, ethnicity, and country of origin, underlying conditions, clinical

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and laboratory data, TB localization and presence of complications, first- and second-line treatment, and side effects recorded during the follow-up. To better assess the role of migration on TB outcome, the patients were classified as follows:

- Italians (born to Italian parents);
- Italians (born to foreign parents): children born in Italy from at least 1 foreign parent;
- Foreign: children born outside Italy from nonItalian parents.

At the end of follow-up, children were classified according to the following outcomes: (1) cure without sequelae, (2) cure following need for prolonged treatment or modification of drug regimen, (3) cure with sequelae, (4) treatment failure/relapse, (5) death, and (6) loss to follow-up.

To analyze the impact of different factors on TB outcomes, all children who did not heal without sequelae were grouped in the composite outcome measure, "poor clinical outcome." Moreover, a separate outcome analysis was performed for patients "lost to follow-up," as belonging to this group does not allow assessment of disease course, and expose children to unfavorable outcome, due to the severity of TB in childhood and the need of a close treatment adherence.

Statistical Analysis

As the PIDU is the unique regional referral center, TB rates were calculated dividing the number of new cases diagnosed at the PIDU with the total number of residents below 18 years of age in the Campania Region, according to the ISTAT. The same source was used to collect data about the number of migrants present in the region during the study period.⁶

Quantitative variables were reported as mean $\pm$ SD, and variables with skewed distributions were presented as median and interquartile range. Results were compared by using t-test or Mann-Whitney nonparametric test, as appropriate. Categorical variables were summarized and reported as frequencies and percentages and compared through Fisher exact test or χ^2 test, as appropriate.

Yearly crude TB incidence rates and relative CIs were calculated adopting the exact method with Poisson distribution (see Table S1, SDC, <http://links.lww.com/INF/E63>). A linear regression model was used to assess the trend of TB incidence during the study period adjusting the model for parents' nationality (Italian/Others), mean age at diagnosis (in months), and TB outcome (healed within expected time and using standard therapy/other outcome).

To better investigate the role of time and migration, the data were successively analyzed according to the year of observation (2009–2013 versus 2014–2018) and patients' origin (Italian versus Foreign). The latter category included either children born outside Italy or those born in Italy to foreign parents.

Univariable analysis of variables influencing the clinical outcome was performed using the logistic regression analysis. Results were reported as crude and adjusted odds ratio (OR) with 95% CIs. Multivariable analysis included age, gender, country of origin, TB localization, drug resistance pattern, results of culture and smear, and variables found to have $P < 0.1$ in the univariable analysis. Two-sided P values < 0.05 were considered statistically significant. Statistical analysis was performed using the Stata MP 15 (StataCorp) and SPSS 21.0 Software (SPSS Inc., Chicago, IL).

Ethical Statement

The study was conducted according to the principles expressed in the Declaration of Helsinki. Patients' data were analyzed and reported anonymously, and caregivers signed a specific-informed consent as in previous publications^{7,8} (see Text 2, SDC; <http://links.lww.com/INF/E63>).

RESULTS

A total of 146 children (76; 52.1% male; median age, 50 months; interquartile range, 96.33) received a diagnosis of TB during the study period, with an increase in the number of cases registered in the period 2014–2018 (90; 61.6%) compared with 2009–2013 (56; 38.4%).

The source of infection was not detected in 51 cases (34.3%); however, when the index case was identified, households were the main source of infection in the majority of the study population: parents in 37 cases (25.3%) and other household members in 18 cases (12.3%) (Table 1). In 29 patients (19 Italians and 10 foreign), the source of infection was identified in nonhousehold close relatives (grandparents or cousins in most cases) and in other 11 in people outside the family (ie, housekeeper and school).

The majority of children (77%) received a diagnosis of pulmonary TB and about one-fourth of the overall population received a microbiologic TB confirmation (Table 1).

No significant differences in the proportion of drug-resistant cases were observed over time (4 cases: 7.1% in 2009–2013 versus 5 cases: 5.5% in 2014–2018).

Although antimicrobial resistances were demonstrated in only 6.2% of overall cases, $< 60\%$ of children showed a complete clinical response to first-line antitubercular treatment (Table 1). A significant difference in the clinical outcome was observed in the study period with a dramatic reduction in the proportion of cases reporting a good clinical outcome from 85.7% to 42.7% ($P < 0.0001$), mainly due to a relevant increase in the proportion of patients who needed a prolonged treatment or a modification of drug regimen (Table 1).

Changes in standard TB treatment timing or regimen were introduced based on clinical, radiologic, or microbiologic findings. The majority of these patients showed a persistence of active lesions on chest X ray (19, 61.3%) or on CT scan (12, 38.7%), and only 5 (16.1%) children showed a lack of relevant clinical improvement (ie, poor weight gain, persistence of fatigue, persistence of lymphadenopathy) during follow-up.

In addition, the lack of a microbiologic confirmation and resistance pattern (22, 71%), as well as the poor adherence to follow-up visits and medical indications in most of these children (17, 54.8%), had an impact in clinical decision processes.

TB Rate and Association With Migration

TB incidence rates increased from 0.44 cases (95% confidence interval [CI]: 0.16–0.97) per 100,000 inhabitants younger than 18 years in 2009 to 1.84 cases (95% CI: 1.15–2.79) in 2018 ($P < 0.05$) (Fig. 1).

In the same period of observation, the percentage of population younger than 18 years who identify as migrants in the Campania Region progressively increased from 1.67% of the total population in 2009 to 3.74% in 2018, according to the data reported by the ISTAT.

We found a highly significant linear correlation between TB incidence rate and rate of migrants in Campania ($R^2 = 0.9272$; $P < 0.0001$). When controlling for age at diagnosis, parents' nationality, and TB outcome, the increase in TB incidence rate was 0.10 (0.03–0.18) per year.

Although the majority of children enrolled in the study (78, 53.4%) were Italian, the number of foreign children and Italians born to foreigner increased over time (Fig. 2). The country of origin of foreign children are reported in Table 2; most foreign children came from high-TB prevalence countries in Eastern Europe, North-Africa, or South Asia.

The source of infection significantly varied according to patients' origin, being more commonly identified among parents

TABLE 1. Characteristics of Patients According to Year of Diagnosis and Geographical Origin

Parameters	Total	2009–2013	2014–2018	<i>P</i>	Italian	Foreign	<i>P</i>
Number of patients (%)	146 (100)	56 (38.4)	90 (61.6)	–	78 (53.4)	68 (46.6)	–
Source case							
Parents (%)	37 (25.3)	14 (25)	23 (25.6)	0.363	13 (16.3)	24 (35.3)	0.038
Household member, non parent (%)	18 (12.3)	10 (17.9)	8 (8.9)		12 (15.4)	6 (8.8)	
Nonhouseholds (%)	40 (27.4)	17 (30.3)	23 (25.6)		26 (30.3)	14 (20.3)	
Unknown (%)	51 (34.3)	15 (26.8)	36 (40)		27 (34.6)	24 (35.3)	
Case definition							
Microbiologically confirmed (%)	39 (26.7)	16 (28.6)	23 (25.6)	0.689	20 (25.6)	19 (27.9)	0.754
Microbiologically unconfirmed (%)	107 (73.3)	40 (71.4)	67 (74.4)		58 (74.4)	49 (72.1)	
Ziehl-Neelsen-positive (%)	15 (10.3)	1 (1.8)	14 (15.6)	0.008	7 (9.0)	8 (11.8)	0.580
PCR positive (%)	32 (21.9)	12 (21.4)	20 (22.2)	0.910	16 (20.5)	16 (23.5)	0.661
Culture positive (%)	27 (18.5)	10 (17.9)	17 (18.9)	0.876	13 (16.7)	14 (20.6)	0.543
Drug resistance							
Sensitive to first-line treatment (%)	137 (93.8)	52 (92.9)	85 (94.4)	0.341	75 (96.2)	62 (91.2)	0.296
Isoniazid or rifampicin resistance (%)	5 (3.4)	1 (1.8)	4 (4.4)		1 (1.3)	4 (5.9)	
MDR (%)	3 (2.1)	2 (3.6)	1 (1.1)		2 (2.6)	1 (1.5)	
PreXDR (%)	1 (0.7)	1 (1.8)	0 (0)		0 (0)	1 (1.5)	
Primary localization							
Pulmonary (%)	112 (76.7)	44 (78.6)	68 (75.6)	0.675	60 (76.9)	52 (76.5)	0.949
Extra pulmonary (%)	34 (23.3)	12 (21.4)	22 (24.4)		18 (23.1)	16 (23.5)	
Main organ involvement							
Pulmonary (%)	107 (74.8)	42 (76.4)	65 (73.9)	0.488	56 (73.7)	51 (76.1)	0.519
Pleuropulmonary (%)	10 (7.0)	3 (5.5)	7 (8)		7 (9.2)	3 (4.5)	
Bronchial/laryngeal (%)	2 (1.4)	1 (1.8)	1 (1.1)		2 (2.6)	0 (0)	
Lymph node (%)	10 (7.0)	2 (3.6)	8 (9.1)		5 (6.6)	5 (7.5)	
Osteoarticular (%)	5 (3.5)	3 (5.5)	2 (2.3)		3 (3.9)	2 (3.0)	
Intestinal (%)	3 (2.1)	0 (0)	3 (3.6)		1 (1.3)	2 (3.0)	
Miliary/CNS (%)	6 (4.2)	4 (7.2)	2 (2.2)		2 (2.6)	4 (6.0)	
Clinical outcome							
Cure without sequelae (%)	86 (59.3)	48 (85.7)	38 (42.7)	0.000	53 (67.9)	33 (49.3)	0.000
Cure following need for prolonged treatment or modification of drug regimen (%)	31 (21.4)	1 (1.8)	30 (33.7)		17 (21.8)	14 (20.9)	
Cure with sequelae (%)	5 (3.4)	2 (3.6)	3 (3.4)		4 (5.1)	1 (1.5)	
Treatment failure/relapse (%)	6 (4.1)	1 (1.8)	5 (5.6)		4 (5.1)	2 (3.0)	
Loss to follow-up (%)	15 (10.3)	3 (5.4)	12 (13.5)		0 (0)	15 (22.4)	
Death (%)	2 (1.4)	1 (1.8)	1 (1.1)		0 (0)	2 (3.0)	

CNS, Central Nervous System; MDR, multiple drug resistant; PCR, polymerase chain reaction; XDR, extensively drug resistant.

and other households in foreign patients rather than in Italian children (Table 1). Comparing Italian and foreign children, no difference in TB localization and rate of microbiologic confirmation was observed. The rate of drug resistance in foreign children (6 cases, 8.8%) was higher than that reported in Italians (3 cases, 3.8%), although this difference did not reach statistical significance (χ^2 , 1.55; $P = 0.212$).

Clinical Outcomes and Loss to Follow-up

Overall, 86 patients (59.3%) undergoing standard antitubercular treatment in accordance with TB localization, severity, and resistance, showed a complete resolution without sequelae (Table 1). About 20% of children needed a prolonged treatment or drug shift to resolve TB, and a minority showed permanent sequelae: 2 had vertebral collapse after TB spondylitis, 2 had permanent atelectasis after severe lung involvement, 1 had a residual frontal lobe tuberculoma after TB encephalitis.

According to univariable logistic regression analysis (see Table S3, SDC; <http://links.lww.com/INF/E63>), a poor clinical outcome was significantly related to TB localization (OR, 2.26; 95% CI: 1.01–5.01; $P = 0.045$), positivity of Ziehl-Neelsen (OR, 5.70; 95% CI, 1.82–17.8; $P = 0.003$) or polymerase chain reaction (OR, 2.58; 95% CI: 1.14–5.82; $P = 0.022$) on gastric aspirate or sputum smear. When regression analysis was adjusted for age, gender, origin, localization of TB, presence of drug resistance, results of culture and smear, only Ziehl-Neelsen-positive patients (OR, 4.83; 95% CI: 1.28–18.2; $P = 0.020$) had increased likelihood of poor

clinical outcome (see Table 3, SDC, <http://links.lww.com/INF/E63>).

Compared with Italians, foreign children showed a significant lower likelihood of cure without sequelae (49.3% versus 67.9%; $P < 0.001$; OR, 0.45; 95% CI: 0.23–0.89; $P = 0.02$). Of note, all fatal cases and loss to follow-up were observed in foreign patients (Table 1).

The risk of an unfavorable TB outcome was not related to a poor disease course (OR, 0.82; 95% CI: 0.40–1.67; $P = 0.589$), but significantly to the loss to follow-up (OR, 49.3; 95% CI: 2.89–840.4; $P = 0.007$) (see Table 3, SDC, <http://links.lww.com/INF/E63>). The likelihood of loss to follow-up was similar in Italian children born to foreign parents (OR, 46.8; 95% CI: 2.61–837.6) and in foreign children (OR, 46.1; 95% CI: 2.54–837.0), when compared with Italians (Table 3).

DISCUSSION

The European Centre for Diseases Control and Prevention classified Italy as a low TB prevalence country, with an overall notification rate of 6.5/100,000 and a rate of about 2/100,000 subjects younger than 15 years of age.² However, the Italian Ministry of Health did not release official TB data in the last 10 years, and the crude notification rate reported to European authorities does not reflect the different situations of single regions.

We observed a significant increase in the TB notification rate in pediatric population resident in Campania Region in the last

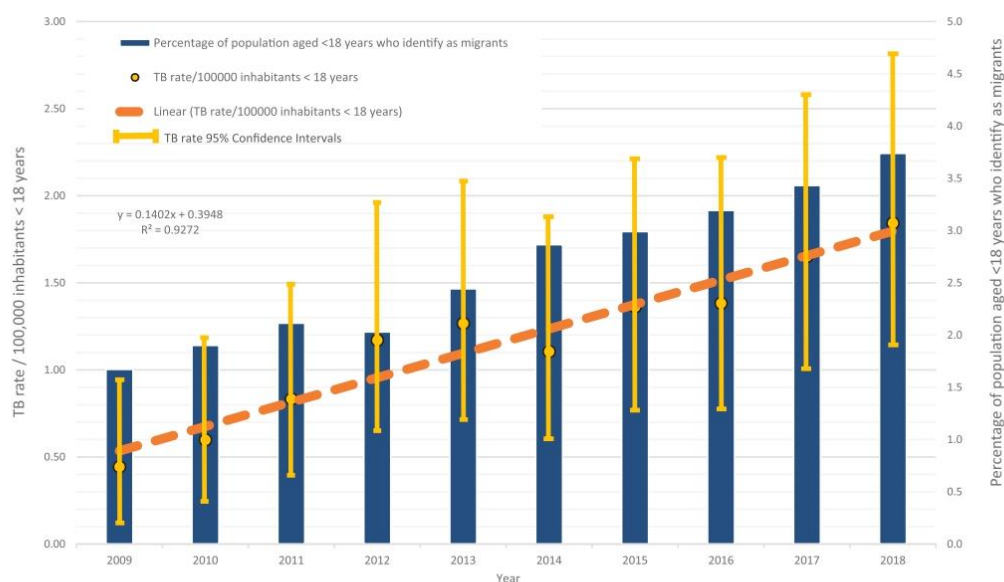


FIGURE 1. Correlation between TB rates and percentage of population <18 years of age who identify as migrants in Campania Region. (i) TB rates are normalized per 100,000 inhabitants below 18 years of age. (ii) The number of migrants is expressed as rate of the total regional population. [full color online](#)

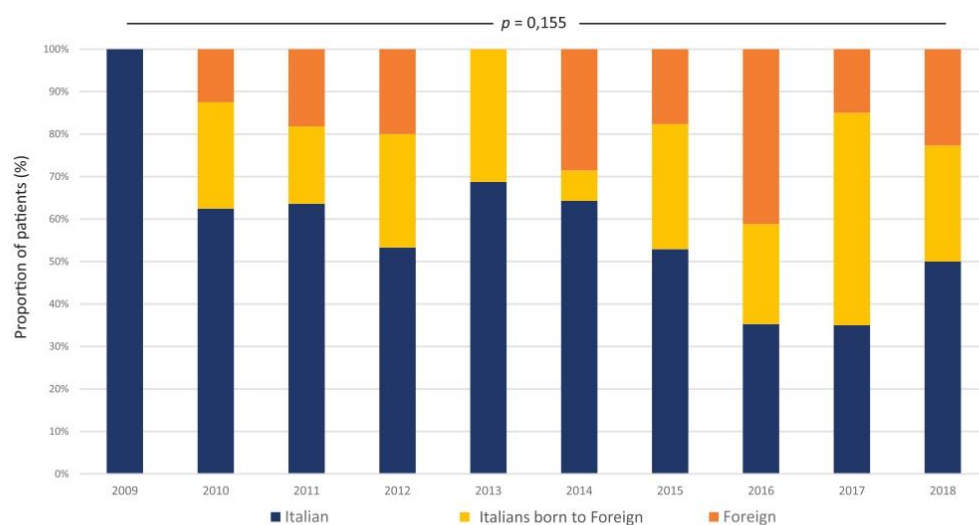


FIGURE 2. Distribution of TB-affected children according to origin and time of diagnosis. [full color online](#)

10 years. This increase may be related to several factors, including (1) the suppression of local prevention measures, (2) the Bacillus Calmette–Guérin shortage, not available even for potential

candidates such as neonates to TB-affected mothers or exposed healthcare workers, (3) a larger use of immunosuppressive treatments, and (4) a progressive change in the local population due to

TABLE 2. Country of Origin of 146 TB-Infected Children

Country of origin	Number of Cases (%)	TB Prevalence Rate ^a
Italy	78 (53.4)	Low
Romania	12 (8.2)	High
Morocco	9 (6.2)	High
Ukraine	7 (4.8)	High
Nigeria	7 (4.8)	High
China	2 (1.4)	High
India	2 (1.4)	High
Somalia	2 (1.4)	High
Albania	2 (1.4)	Intermediate
Bangladesh	1 (0.7)	High
Benin	1 (0.7)	High
Brazil	1 (0.7)	Intermediate
Burundi	1 (0.7)	High
Eritrea	1 (0.7)	High
Pakistan	1 (0.7)	High
Paraguay	1 (0.7)	Intermediate
Poland	1 (0.7)	Intermediate
Republic of Côte d'Ivoire	1 (0.7)	High
Russia	1 (0.7)	High
Serbia	1 (0.7)	Intermediate
Somalia	1 (0.7)	High
Unknown	14 (9.6)	

^aWorld Health Organization TB incidence rates (available online) were used to stratify based on country of origin: "low" TB risk (<10/100,000 inhabitants), "intermediate" TB risk (10–50/100,000), and "high" TB risk (≥50/100,000).

migration of young adults seeking jobs in Northern Italy or abroad, and permanence of children, elderly or unprivileged people, generally considered at higher risk for TB.

In parallel with the escalation of pediatric TB notification rate in Campania, the ISTAT reported an increase in migration of subjects below 18 years of age. Although our analysis demonstrated a close linear correlation between the 2 phenomena, a causation cannot be established.

Campania Region currently hosts more than 250,000 migrants, accounting for about 5% of local population, and its harbors receive a large number of asylum seekers who spread-out all-around Europe within a few days or months after arrival. In our population, the rate of foreign increased over time and most migrants came from a high or intermediate TB prevalence area, according to World Health Organization's classification.⁹ The percentage of children diagnosed with TB who were of foreign origin was significantly higher than that reported in other Italian regions; Clemente et al reported about 10% of migrants in a pediatric TB population in Sardinia.¹⁰ However, Campania is "border area" between Africa and Middle East and the rest of Italy and Europe and endures a bottle-neck effect, with a high number of migrants arriving and being distributed all over the continent.

Recent trends show that the majority of TB cases in Western European countries occur in individuals of foreign origin,¹¹ whose

screening varies according to national guidelines. Most national public health agencies (including United Kingdom, Canada, Australia, Ireland) recommend the screening of subjects below 16 years of age coming from intermediate-to-high TB prevalence areas.^{12–15} Italian guidelines¹⁶ recommend to seek signs and symptoms of TB in all migrants arriving in Italy and screen subjects coming from high prevalence countries for LTBI, but this practice is frequently disregarded.

Some countries reporting cases of pediatric TB in asylum seekers recently proposed to review current indications, improving active TB screening among children, and introducing large LTBI screening for asylum seekers <18 years of age.^{17,18} In a recent school-based program in a TB low-prevalence country, no cases of active TB and only 17 of LTBI were identified in >1000 migrant children.¹⁹ Italian regions that applied different screening policies reported a TB prevalence rate ranging from 0.17% in a large Sicilian harbor to 1.8% in Lombardia region; however, rates are not differentiated by age.²⁰

Families seeking asylum have multiple stressors and competing priorities, which may compromise their ability to comply with country-specific models of care. As highlighted by Aldridge,²¹ the TB screening should be considered as "part of a broad package of measures" in which preentry screening needs to be integrated with other initiatives that address the health needs of migrants, as well as other nonmedical stressors for the family.

Foreign families have a more difficult access to healthcare facilities, language barriers, a reduced perception of TB risks, and a higher proportion of missed medical visits than Italian families.⁸ Those barriers may delay TB diagnosis and affect compliance to treatment and follow-up. In a large observational study in children living in United States, the average time from arrival in the hosting country and TB diagnosis varied between 6 months and 4 years according to the country of origin.²²

In our population, foreign children showed a lower likelihood of cure without sequelae, and, notably, they accounted for all fatal cases. If compared with Italians, either foreign children or Italian born to foreign parents had a significantly higher risk to loss of follow-up. Of note, Italian and foreign children with appropriate follow-up showed similar clinical outcome.

Those data support the need of defining specific healthcare programs for children coming from endemic areas, including a support by social workers, an interpreters' collaboration, interaction with outpatient's clinics and pharmacies to ensure adequate drug distribution and assumption, and appropriate follow-up.

A previous multicenter Italian study⁸ attributed the risk of poor TB outcome in children from foreign families to a high rate of multidrug-resistant strains. Apparently, this aspect had a limited impact in our population. Drug resistance was demonstrated in 6% of microbiologically confirmed cases and had a similar distribution over time and according to patients' origin.

This study has some limits. First, the notification rate was calculated on subjects <18 years of age and might be not comparable with that reported by European authorities that refers to subjects

TABLE 3. Distribution of Poor Clinical Outcome and Loss to Follow-up in TB Patients According to Child Origin

Child Origin	Clinical Outcome					Follow-up				
	Favorable n (%)	Poor n (%)	P	OR	95% CI	Completed follow-up n (%)	Lost to follow-up n (%)	P	OR	95% CI
Italians born to Italian parents	53 (67.9)	25 (32.0)	0.764	—	—	78 (100)	0 (0)	<0.0001	—	—
Italians born to foreign parents	18 (66.6)	9 (33.3)		1.06	0.41–2.68	28 (77.7)	8 (22.3)		46.8	2.61–837.6
Foreign	15 (60.0)	10 (40.0)		1.41	0.55–3.58	25 (78.1)	7 (21.8)		46.1	2.54–837.0

Italians born to Italian parents were used as reference category.

<15 years of age. However, our reference center manages children up to 18 years of age and only 5 of those included in the study had an age between 15 and 18 years. Second, some of the cases who required a prolonged or add-on therapy may have been misinterpreted due to the low rate of microbiologic confirmation. Finally, the high rate of loss to follow-up in foreign children (20%) does not allow an accurate evaluation of clinical outcomes in this population. However, we are aware that children not closely monitored and appropriately treated are exposed to severe consequences or even lethality; hence, we would consider the loss to follow-up as an unfavorable clinical outcome.

In conclusion, although the Italian TB rate is stable or slightly reduced in the last years, the TB notification rate in Campania Region has increased in the last 10 years in parallel with the increase in migration rate. Emphasizing national TB rates may disregard important differences in local infection trends, limit medical awareness about the problem, and dismantle local prevention measures. Children from foreign families have a lower probability of cure without sequelae and a higher risk of loss to follow-up and may need specific healthcare programs.

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RESEARCH



Effects of COVID-19 pandemic on pediatric tuberculosis: decrease in notification rates and increase in clinical severity

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Abstract

The outbreak of COVID-19 resulted in a decrease in tuberculosis notification rates globally. We compared tuberculosis incidence rates and disease severity in children seen in our centre prior and during COVID-19 pandemic. We performed a cohort study enrolling children aged under 18 years who received a diagnosis of tuberculosis (January 1st, 2010–December 31st, 2021) at our Pediatric Infectious Diseases Unit. Disease severity was evaluated based on: the classification proposed by Wiseman et al., smear positivity, presence of symptoms at presentation, lung cavitation, extrapulmonary disease, respiratory failure and need for intensive care support. Overall, 168 children (50.6% female, median age 69 months, IQR 95.4) received a diagnosis of tuberculosis, 156 (92.8%) between 2010–2019, before COVID-19 outbreak, and 12 (7.2%) between 2020–2021, during the pandemic. The annual tuberculosis notification rate dropped by 73% in 2021 (0.38/100000, 95%CI 0.1–0.96) compared with 2019 (1.46/100000, 95%CI 0.84–2.37). Compared to the pre-pandemic period, the proportion of children classified as severe was higher in 2020–2021 (5, 41.6% vs 23, 15.7%, $p=0.006$) with a higher rate of respiratory failure (2, 16.7%, vs 4, 2.6%, $p=0.01$) and an increased need for intensive care support (1, 8.3% vs 1, 0.6%, $p=0.01$).

Conclusion: During COVID-19 pandemic we observed a reduction in tuberculosis notification rate in pediatric population and a significant increase in disease severity. This scenario may be the consequence of a delay in diagnosis and an under-reporting of cases, rather than the effect of a reduced transmission of tuberculosis. Children reached health-care services only in the need of urgent medical attention.

What is Known:

- COVID-19 pandemic had a huge impact on national health care systems, resulting in a reduction of access to medical care.

What is New:

- In Campania Region, Italy, a low tuberculosis incidence country, we witnessed a 75% reduction in tuberculosis notification rate during pandemic. In parallel we demonstrated a significant increase in disease severity, suggesting that the reduction in notification rate may be attributed to an underreporting of cases and consequential diagnostic delay, rather than a reduced transmission of infection.

Keywords Tuberculosis · Children · Infection rate · Disease severity · COVID-19

Abbreviations

COVID-19	2019-CoronaVirus Disease
SARS-CoV-2	Severe Acute Respiratory Syndrome CoronaVirus 2
TB	Tuberculosis

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Introduction

The outbreak of Coronavirus Disease (COVID-19) resulted in a decrease in tuberculosis (TB) notification rates globally.

As a reference center for pediatric TB in a Southern Italy populous region, we observed a drop in TB diagnoses that was countertrend compared to the last ten years of observation [1]. This reduction in TB notification might be the consequence of an underreporting of cases and a delay in diagnosis, that could have affected disease severity at presentation and clinical outcome.

To test this hypothesis, we hereby compare TB incidence rates, disease severity and outcome in children seen prior and during COVID-19 pandemic.

Methods

We performed a single center cohort study enrolling all children (< 18 years) who received a microbiologically confirmed or unconfirmed diagnosis of TB between January 1st, 2010 and December 31st, 2021 at our Pediatric Infectious Diseases Unit. Data was collected by using a prospective database as previously reported [1].

TB disease was defined according to established consensus criteria [2]: i) confirmed TB (microbiological confirmation by culture or polymerase chain reaction), or ii) unconfirmed TB: presence of at least two of the following: a) symptoms/signs suggestive of TB, b) chest radiograph consistent with TB, c) known TB exposure or immunologic evidence of TB infection, d) positive response to TB treatment. Children exposed to TB or diagnosed with latent TB infection were excluded. Patients were classified as affected by pulmonary or extrapulmonary TB according to the European Centre for Disease Prevention and Control criteria. Those with multiple disease localizations were classified considering the most severe clinical manifestation.

Disease severity was evaluated based on the following: the classification proposed by Wiseman et al. according to localization, extent and complexity of TB disease [3], smear positivity, presence of symptoms at presentation, lung cavitation, extrapulmonary disease, respiratory failure and need for intensive care support. The definition of poor clinical outcome was based on the need of a prolonged TB treatment (at least one month beyond usual indications), the presence of sequelae, relapse and death.

As the PIDU is the unique regional referral center, TB rates were calculated dividing the number of new cases diagnosed at the PIDU with the total number of residents below 18 years of age in the Campania Region, according to the Italian Institute of Statistics.

Results were compared by using t-test or MannWhitney nonparametric test, as appropriate. Categorical variables were summarized and reported as frequencies and percentages and compared through Fisher exact test or χ^2 test, as appropriate.

Results

Overall 168 children (50.6% female, median age 69 months, IQR 95.4) received a diagnosis of TB during the study period, 156 (92.8%) between 2010–2019, before COVID-19 outbreak, and 12 (7.2%) between 2020–2021, during the

pandemic (Table 1). The majority of children (128, 76.2%) had pulmonary TB, with no significant difference between the two periods (58.3% vs 77.5%, $p=0.13$), and 38 (22.6%) had microbiological confirmation.

The mean number of incident cases per year decreased from 15.6 ± 4.0 in the pre-pandemic period to 6 ± 2.8 in 2020–2021 ($p=0.058$), and the annual TB notification rate dropped by 73% in 2021 (0.38/100000, 95%CI 0.1–0.96) compared with 2019 (1.46/100000, 95%CI 0.84–2.37).

During the pandemic a higher proportion of children came to testing for TB and received diagnosis because of the onset of symptoms (7, 58.3%) compared to the two years pre-pandemic 2018–2019 (7, 18.4%, $p=0.007$), without reaching statistical significance when compared to 2010–2019.

The proportion of children classified as severe was higher in 2020–2021 (5, 41.6%) compared with 2010–2019 (23, 14.7%, $p=0.006$) (Table 1).

Compared to the pre-pandemic period, children diagnosed with TB during COVID-19 pandemic showed a higher rate of respiratory failure (2, 16.7%, vs 4, 2.6%, $p=0.01$), an increased need for intensive care support (1, 8.3% vs 1, 0.6%, $p=0.01$) and a higher proportion of poor clinical outcome (7, 58.3% vs 48, 30.7%, $p=0.04$).

The two-years pre-pandemic (2018–2019) comparative analysis confirmed a statistically significant increase in the proportion of severe TB cases during the pandemic (2020–2021) (Table 1), although the rates of respiratory failure, intensive care admission and poor outcome did not reach statistical significance.

Children receiving a TB diagnosis during COVID-19 pandemic had a significantly higher risk of presenting with severe disease, compared with those diagnosed in 2010–2019 (OR 3.93, 95%CI 1.15 to 13.4, $p=0.028$) and also with those diagnosed in the last two years before the pandemic (OR 6.07, 95%CI 1.29 to 28.5, $p=0.022$).

Discussion

During COVID-19 pandemic a marked decrease in TB notification rate has been reported at European [4] and global level [5]. However, the effects that pandemic restrictions had on children living in European countries are unknown.

The present study revealed a 70-percent drop in TB incident cases among children living in a low-incidence European Country, and demonstrated a parallel rise in the severity of the disease during the first two years of COVID-19 pandemic.

The decrease in the number of new TB diagnoses could have been either the effect of a reduced transmission of TB, as an effect of Severe Acute Respiratory Syndrome CoronaVirus 2 (SARS-CoV-2) containment measures, or the consequence of a delay in diagnosis and an underreporting of cases.

Table 1 Incidence, clinical features and outcomes of children with TB (n=168) in pandemic and pre-pandemic period

	Pandemic (2020–2021) TB cohort	Pre-pandemic (2010–2019) TB cohort	<i>p</i>	2-year pre-pandemic (2018–2019) TB cohort	<i>p</i>
TB incident cases	12	156	-	38	-
TB rate/100.000 inhabitants (95%CI)	0.56 (0.01–1.25)	1.17 (0.24–2.10)	-	1.66 (0.49–2.83)	-
Mean number of cases/year (SD)	6.0 (2.8)	15.6 (4.0)	0.058	19.0 (4.3)	0.042
Female gender (%)	7 (58.3)	78 (50.0)	0.577	22 (57.8)	0.978
Foreign origin (%)	9 (75.0)	77 (49.3)	0.086	20 (52.6)	0.171
Reason for TB testing					
Contact tracing	5 (41.7)	99 (63.4)	0.13	29 (76.3)	0.02
Symptoms	7 (58.3)	53 (34.0)	0.08	7 (18.4)	0.007
Migrant screening/adopted children	0 (0.0)	4 (2.6)	1.00	2 (5.3)	1.00
Case definition					
Microbiologically confirmed (%)	4 (33.3)	34 (21.7)	0.357	13 (34.2)	0.358
- Ziehl–Neelsen-positive (%)	1 (8.3)	13 (8.3)	1.00	6 (15.8)	0.516
- Gene-X-pert-positive (%)	4 (33.3)	33 (21.1)	0.005	12 (31.5)	0.909
- Culture positive (%)	2 (16.7)	23 (14.7)	0.856	8 (21.0)	0.740
Drug resistance					
Sensitive to first-line treatment (%)	12 (100.0)	146 (93.5)	1.00	36 (94.7)	1.00
Isoniazid/Rifampin resistant (%)	0 (0.0)	5 (3.2)	1.00	1 (2.6)	1.00
Multi drug resistant (%)	0 (0.0)	5 (3.2)	1.00	1 (2.6)	1.00
Primary localization					
Pulmonary (%)	7 (58.3)	121 (77.5)	0.131	32 (84.2)	0.059
Pleuro-pulmonary (%)	3 (25.0)	10 (6.4)	0.020	2 (5.3)	0.046
Bronchial/laryngeal (%)	0 (0.0)	2 (1.3)	1.00	0 (0.0)	1.00
Lymph Nodes (%)	1 (8.3)	10 (6.4)	0.795	3 (7.9)	0.160
Osteo-articular (%)	0 (0.0)	4 (2.6)	1.00	0 (0.0)	1.00
Intestinal (%)	0 (0.0)	3 (1.9)	1.00	0 (0.0)	1.00
Miliary/meningeal (%)	0 (0.0)	6 (3.8)	1.00	1 (2.6)	1.00
Other (%)	1 (8.3)	0 (0.0)	1.00	0 (0.0)	1.00
Disease severity					
Severe cases according to Wiseman's classification (%)	5 (41.6)	23 (14.7)	0.006	4 (10.5)	0.014
Symptoms at presentation (%)	7 (58.3)	102 (65.4)	0.621	24 (63.1)	0.764
Smear-positive (%)	1 (8.3)	13 (8.3)	1.00	6 (15.8)	0.516
Lung cavitation (%)	2 (16.7)	8 (5.1)	0.104	3 (7.9)	0.377
Respiratory failure (%)	2 (16.7)	4 (2.6)	0.011	1 (2.6)	0.074
Extra-pulmonary disease (%)	5 (41.6)	35 (22.4)	0.131	6 (15.8)	0.059
Need for intensive care support (%)	1 (8.3)	1 (0.6)	0.017	0 (0.0)	0.072
Clinical outcome					
Healing	5 (41.6)	89 (57.0)	0.300	17 (44.7)	0.851
Poor outcome	7 (58.3)	48 (30.7)	0.049	16 (42.1)	0.325
- Need for prolonged treatment/drug shift (%)	6 (50.0)	34 (21.8)	0.027	10 (26.3)	0.125
- Sequelae (%)	1 (8.3)	6 (3.8)	0.453	3 (7.9)	0.160
- Relapse (%)	0 (0.0)	6 (3.8)	1.00	3 (7.9)	1.00
- Death (%)	0 (0.0)	2 (1.3)	1.00	0 (0.0)	1.00
Lost to follow-up	0 (0.0)	19 (12.1)	0.364	5 (13.1)	0.319

In Italy the overall TB notification rate decreased by about one third in 2020 [4], which could reflect COVID-19 containment measures, such as social distancing and face masks, leading to lower TB transmission in the community. Before the pandemic, mask use among TB patients was poor, due to the fear of stigma, and only 2% of drug-resistant TB patients reported wearing surgical masks in public areas [6].

The effect of reduced transmission in the community is likely to be observed earlier in children, who usually progress faster to TB disease than adults following primary *M. tuberculosis* infection. On the other hand, staying at home could have accelerated transmission within households, an important aspect in children for whom the index case is typically an adult household member.

During the pandemic, children had reduced access to medical care due to the parents' fear of being infected with SARS-CoV-2 in hospital settings, to the barriers towards direct visits by primary care physicians, and to the reallocation of health-care personnel toward the COVID-19 units. Migliori et al. reported a reduction of TB services based on data collected from 33 centers located in 16 countries worldwide [7]. McQuaid CF et al. suggested that, although social distancing measures can be beneficial in settings where health services are less affected, resulting in a lower number of TB cases, this beneficial effect is more likely to be outweighed by health service disruption, especially in low- and middle-income settings [8].

In Campania region, a populous region in Southern Italy, we previously observed a significant and progressive increase in TB notification rates between 2009 and 2018. This phenomenon was directly associated with migration [1]. Notably, the drop in TB diagnosis observed during the pandemic appeared unrelated to migration flows in the region, where the rate of migrants concomitantly increased, reaching 4% of the resident pediatric population [9]. During the pandemic, the risk of TB exposure in migrants was limited by restrictions to visit friends and relatives in high TB incidence countries, however we actually observed a high proportion of children of foreign origin among TB diagnoses. While this could be related to a lack of Italian TB cases, the magnified difficulties in accessing medical care for migrant populations must be taken into account and the number of TB cases of foreign origin might even be underestimated.

In addition, for children seeking care for respiratory symptoms and fever, the priority was to exclude SARS-CoV-2 infection, rather than performing detailed diagnostics for other infections, including TB.

The World Health Organization estimated that the decrease in TB case detection could result in half a million excess TB deaths in the next few years, depending on the degree and duration of disruption in disease detection [10].

During the pandemic, children often reached health services only when they were severely ill and required urgent medical attention, and the few who received a new diagnosis of TB showed compromised clinical conditions and a largely disseminated TB infection. According to our data, receiving a diagnosis during COVID-19 pandemic exposed to a 3- to sixfold increased chance of severe TB disease.

Of note, none of the new patients receiving a diagnosis of TB resulted positive to SARS-CoV-2 tests at admission, and we did not observe any severe SARS-CoV-2 infection requiring hospital care in children in follow-up for TB disease.

No major differences in drug resistance pattern, access to specific treatment or adherence to follow-up were observed in the cohorts.

Notably, some centers reported increased follow-up losses during pandemic [11]. We actually observed that the main barrier to hospital access for new TB patients was related to caregivers' hesitancy to reach the hospital during pandemic, rather than being related to a shortage of health-care personnel. In our institution, the same specialists who took care of COVID-19 patients were involved in the management of children with other infections (including TB patients managed in a different hospital area). Hence, we have been able to keep follow-up service active during pandemic, rescheduling any missed appointments as soon as possible.

This supports our hypothesis that the delay in TB diagnosis and the underreporting are the major determinants of the increased disease severity and more severe clinical outcome observed in our population.

Although in 2021 most restrictive measures were dismantled and TB services were completely restored in the region, the TB notification rate was even lower than that in 2020. This indicates that the restoration of standard services might be not sufficient, and it raises a worrisome question: "how long would it take to track and treat all undetected cases of TB that we missed during the pandemic?".

Supplementary measures are urgently required to attenuate the impact of COVID-19 pandemic in order to catch-up with the missed diagnoses.

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Data availability The corresponding author is responsible for the collection of data, their anonymization, management and presentation.

The entire database of the present study is available on appropriate and motivated request.

Declarations

Ethical approval The study was conducted in accordance with the 1964 Helsinki Declaration and its later amendments, and with the ethical standards of the institutional research committee (Ethic Committee University “Federico II”). Patient’s data were extrapolated from a pre-existing database created in 2009 and used to prospectively record data of children with TB seen at the PIDU. The Campania Reference Centre’s dataset is part of the Italian Register of Paediatric Tuberculosis, a larger national database created and implemented by the Italian Society of Paediatric Infectious Diseases and approved by the Institutional Review Board of the University of Florence. The same database was previously used to report trends in the local paediatric population [1]. The present analysis did not undergo new formal approval by the Ethical Committee. All patients’ data were registered, analyzed and reported anonymously.

Informed consent Legal guardians signed a specific informed consent to collect and process data.

Competing interests The authors have no relevant financial or non-financial interests to disclose.

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CHAPTER 3

Diagnostic Challenges in Pediatric TB: Evidence from the PTBnet Network

Approximately half of all pediatric TB cases remain underreported or undiagnosed, largely due to the considerable diagnostic challenges inherent to this population (1). This substantial diagnostic gap represents a major obstacle to global TB elimination efforts, as undetected and untreated cases continue to sustain transmission and contribute to preventable morbidity and mortality among children (1).

The paucibacillary nature of infection in children, difficulties in obtaining adequate specimens, and the limited sensitivity of immune-based assays represent the main microbiological and immunological barriers to timely and accurate diagnosis (2,3,4).

These limitations are even more pronounced in disseminated forms and in immunocompromised children, who are at higher risk of severe outcomes (5).

The two multicenter studies presented here, conducted within the framework of the Pediatric TB Network European Trials Group (PTBnet), provide complementary perspectives on the performance of diagnostic immunological tools in pediatric populations, including immunocompromised children, and underline the complexity of disease recognition in these vulnerable groups. PTBnet (Pediatric TB Network European Trials Group) is the largest collaborative research network in Europe dedicated to pediatric TB and supported by European Society for Paediatric Infectious Diseases (ESPID). It brings together more than one hundred clinicians and researchers from about 20 countries and maintains a shared centralized, REDCap-based multicentre database. By enabling the prospective and retrospective collection of standardized clinical, laboratory and outcome data across different healthcare settings, PTBnet facilitates collaborative research studies that are essential to generate robust evidence on TB in children (6).

In this context, our centre has actively collaborated with PTBnet, and I performed the clinical data collection that contributed to the analyses underlying both studies, reproduced below.

This local engagement within a large international network underlines how multicentre cooperation is indispensable to advance the evidence base for pediatric TB.

The first study evaluated the performance of the QuantiFERON-TB Gold Plus (QFT-Plus) assay compared with previous interferon-gamma release assays (IGRAs) and the tuberculin skin test (TST) in over 1,000 children across multiple European centers. Contrary to expectations, QFT-Plus did not perform better than earlier IGRAs or the TST in terms of sensitivity, and all tests exhibited particularly poor sensitivity in children with central nervous system or miliary TB and in those with immunocompromise. Importantly, none of the currently available immune-based tests achieved sufficient accuracy to be used as reliable rule-out diagnostics in children, highlighting the urgent need for novel tools (7).

The second study focused specifically on immunocompromised children with TB, a population at heightened risk of severe disease and long-term sequelae. Compared with immunocompetent controls, immunocompromised children presented more frequently with nonrespiratory and disseminated forms of TB and were significantly more likely to develop severe disease. Diagnostic accuracy was markedly reduced in this cohort, with both TST and QFT assays showing high rates of false-negative results. Although mortality was low, the burden of chronic sequelae was significantly higher. Thus, immunocompromised children constitute a double challenge: they are both more susceptible to severe forms of TB and harder to diagnose with existing tools (8).

Taken together, these studies illustrate the limitations of currently available diagnostic strategies for pediatric TB. They confirm that immune-based tests, though widely used, are insufficiently sensitive, particularly in the youngest children, in disseminated disease, and in the setting of immunosuppression, but also in general population.

In summary, the evidence consolidated in these two multicenter studies, and made possible through the unique collaborative structure of PTBnet, reaffirms that pediatric TB cannot be approached as a simple extrapolation of adult disease.

The diagnostic challenges are distinct, compounded in settings of immunological vulnerability, and demand dedicated research efforts to develop sensitive and specific, diagnostic tools. Only through such advances can meaningful progress be achieved toward the global goal of ending TB in children.

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Original research

Performance of QuantiFERON-TB Gold Plus assays in paediatric tuberculosis: a multicentre PTBNET study

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ABSTRACT

Rationale In 2016, a new interferon-gamma release assay (IGRA) was introduced, QuantiFERON-TB Gold Plus (QFT-Plus), claimed to have improved sensitivity in active tuberculosis (TB).

Objectives This study aimed to determine the performance of QFT-Plus, compared with previous generation IGRAs and the tuberculin skin test (TST), in children with TB in Europe.

Methods Multicentre, ambispective cohort study within the Paediatric Tuberculosis Network European Trials Group (ptbnet), a dedicated paediatric TB research network comprising >300 members, capturing TB cases <18 years-of-age diagnosed between January 2009 and December 2019.

Measurements and main results 1001 TB cases from 16 countries were included (mean age (IQR) 5.6 (2.4–12.1) years). QFT-Plus was performed in 358, QFT Gold in-Tube (QFT-GIT) in 600, T-SPOT.TB in 58 and TST in 636 cases. The overall test sensitivities were: QFT-Plus 83.8% (95% CI 80.2% to 87.8%), QFT-GIT 85.5% (95% CI 82.7% to 88.3%), T-SPOT.TB 77.6% (95% CI 66.9% to 88.3%) and TST (cut-off ≥ 10 mm) 83.3% (95% CI 83.3% to 86.2%). There was a trend for tests to have lower sensitivity in patients with military and/or central nervous system (CNS) TB (73.1%, 70.9%, 63.6% and 43.5%, respectively), and in immunocompromised patients (75.0%, 59.6%, 45.5% and 59.1%, respectively).

Conclusions The results indicate that the latest generation IGRA assay, QFT-Plus, does not perform better than previous generation IGRAs or the TST in children with TB disease. Overall, tests performed worse in CNS and military TB, and in immunocompromised children. None of the tests evaluated had sufficiently high sensitivity to be used as a rule-out test in children with suspected TB.

INTRODUCTION

Paediatric tuberculosis (TB) remains a major global health issue, with at least one million children newly

WHAT IS ALREADY KNOWN ON THIS TOPIC

⇒ Diagnosing tuberculosis (TB) in children remains challenging, mainly as children typically have paucibacillary disease. Consequently, paediatricians frequently have to rely on immune-based tests, such as the tuberculin skin test (TST) and interferon-gamma release assays (IGRAs), to support a presumptive diagnosis of TB.

WHAT THIS STUDY ADDS

⇒ The latest generation IGRA assay, QuantiFERON-TB Gold Plus, does not perform better than previous generation IGRAs or the TST in children with TB disease. Overall, tests performed worse in central nervous system and military TB, and in immunocompromised children.

HOW THIS STUDY MIGHT AFFECT RESEARCH, PRACTICE OR POLICY

⇒ None of the currently available immune-based diagnostics have sufficiently high sensitivity to be used as a rule-out test in children with suspected TB. More research and funding are needed to develop new diagnostic tests able to define the TB spectrum in children.

diagnosed with TB each year, representing 12% of all TB cases and resulting in an estimated 230 000 deaths.¹ Despite this large disease burden, paediatric TB continues to receive inadequate attention.²

Achieving a definitive TB diagnosis in children is often challenging due to the lack of specific signs and symptoms, difficulties in obtaining sputum samples and the paucibacillary nature of TB in this age group.³ Therefore, detection and treatment of latent TB infection (LTBI) in children to prevent



progression to TB disease are key strategies for controlling TB.⁴ Importantly, the risk of progression is substantially higher in children than in adults, with approximately 30%–40% of infants and 10%–20% of children with LTBI progressing to TB disease within 2 years after primary infection.⁵

The WHO has pledged to work towards eliminating TB globally by 2035 through the End TB Strategy.⁶ Accurate diagnostic tests, which can detect both LTBI and TB disease early, are critical in that strategy. Traditionally, the tuberculin skin test (TST) has been used for LTBI diagnosis, and is also used as a supportive diagnostic tool in children with suspected TB disease. However, the TST has a number of well-documented limitations, including suboptimal specificity.^{7–8} In recent years, newer immune-based diagnostics to detect TB infection have become available for clinical use, of which interferon-gamma release assays (IGRAs) are the most commonly used in high-resource countries.⁹ Despite early generation IGRAs (ie, QuantiFERON-TB Gold (QFT), QFT Gold in-Tube (QFT-GIT) and T-SPOT.TB) being more specific than the TST, they were found to lack the ability to distinguish between LTBI and TB disease and also to have suboptimal sensitivity in patients with TB disease.^{10–11}

In 2016, the latest generation QFT assay, QFT-Plus (QIAGEN, Germantown, Maryland, USA) was released. In contrast to its immediate predecessor, the QFT-GIT assay, QFT-Plus antigen tubes only contain ESAT-6 and CFP-10 as stimulatory *Mycobacterium tuberculosis* (MTB) antigens, but not TB7.7. Furthermore, QFT-Plus assays have a newly added second antigen tube (TB2) containing shorter ESAT-6 and CFP-10 peptides, aimed at eliciting CD8+ T cell responses, in addition to the first antigen tube (TB1), which contains longer peptides directed at CD4+ T cells.¹²

Data in adults suggest that QFT-Plus may have higher sensitivity than QFT-GIT while maintaining high specificity.¹¹ Moreover, the ability to compare CD4 and CD8 responses has been claimed to potentially aid the distinction between TB disease and LTBI.¹³ However, the existing literature related to QFT-Plus almost exclusively consists of adult studies.^{14–17} Furthermore, there are no published studies that included paediatric patients from a variety of geographical locations. This is an important limitation since many factors, including nutritional status and immunocompromise, have been shown to impair the performance of previous generation IGRAs in children.¹⁸

This study aimed to determine the sensitivity of QFT-Plus assays compared with previous generation IGRAs and the TST in children with both clinically diagnosed and microbiologically confirmed TB disease across a large number of European collaborating centres, and to identify factors that impact on assay performance.

METHODS

This ambispective observational multicentre study was conducted by the Paediatric Tuberculosis Network European Trials Group (ptbnet), which currently includes 304 clinicians and researchers based in 31 European countries. The ptbnet database, hosted at Hospital Gregorio Marañón (Madrid, Spain),¹⁹ was launched in 2017, capturing data from children and adolescents <18 years of age diagnosed with TB disease at participating healthcare centres. Participating centres are able to enter patients that have received care previously (retrospective arm), as well as newly diagnosed patients (prospective arm); the dataset for each patient is identical, irrespective of whether the patient was included retrospectively or prospectively. The precise time point (between launch of the database on 1 May 2017 and study closure on 31 December 2019) when individual centres started to contribute patients prospectively varied between centres, depending on the timing of institutional and ethical approvals.

TB disease was defined according to established consensus criteria: (1) confirmed TB (microbiological confirmation by culture or PCR) or (2) unconfirmed TB: presence of at least two of the following: (A) symptoms/signs suggestive of TB, (B) chest radiograph consistent with TB, (C) known TB exposure or immunologic evidence of MTB infection and (D) positive response to TB treatment.²⁰ The TB disease focus was categorised as follows: 'respiratory TB' comprised individuals with TB disease involving the lung parenchyma, intrathoracic lymph nodes, larynx, trachea, bronchus or pleura without disease elsewhere; 'miliary and/or central nervous system (CNS) TB' comprised children with disseminated TB, children with TB meningitis or parenchymal CNS disease, and those who had both; 'other disease focus' comprised patients with peripheral lymph node, osteoarticular, genitourinary, abdominal and pericardial TB.

Inclusion criteria

Patients were eligible for inclusion if they were <18 years of age at the time when the diagnosis of TB disease was made, were diagnosed during the above-mentioned time period, received care at one of the participating centres and had undergone at least one IGRA test at the time of diagnosis. Patients not fulfilling all four inclusion criteria were excluded from participation.

Diagnostic tests

QFT-GIT, QFT-Plus and T-SPOT.TB assays were performed in fully-accredited diagnostic laboratories at each participating institution, and results were interpreted according to the version of the manufacturer's recommendations valid at the time. QFT-GIT and QFT-Plus results were categorised as positive, negative or indeterminate; where available, quantitative background-corrected QFT antigen responses (ie, TB1-nil or TB2-nil result in IU/mL) were also collected. T-SPOT.TB results were classified as positive (≥ 8 spots), negative or indeterminate. TSTs were performed by intradermal injection of purified protein derivative (RT23; Statens Serum Institut, Copenhagen, Denmark or Tubersol; Sanofi Pasteur, Lyon, France), with results read after 48–72 hours. Considering that the national guidelines of different countries vary in their definition of a positive TST result, only children with available quantitative TST results were included in the analyses related to TST performance; both ≥ 5 mm (TST5) and ≥ 10 mm (TST10) cut-offs were evaluated.

Statistical analyses

Categorical variables are reported as absolute frequencies and percentages; continuous variables are reported as median and IQRs. For each test, the test sensitivity was calculated with 95% CI, considering indeterminate IGRA results as negative. Test performance was determined against two gold standards: (1) confirmed and unconfirmed cases combined (ie, the entire study population), and (2) confirmed TB cases only. Wald χ^2 test and Fisher's exact test were used to compare test performances. To account for multiple statistical comparisons, Bonferroni correction was applied. Quantitative background-corrected antigen-stimulated interferon-gamma (IFN- γ) responses in QFT-Plus and QFT-GIT assays among subgroups, stratified by age and TB disease focus, were compared with Kruskal-Wallis tests. Total agreement and Cohen's kappa coefficient (k) were used to quantify concordance between TST and IGRA results; indeterminate IGRA results were excluded from this particular analysis. All analyses were done with SPSS (V.25; IBM SPSS Statistics: Armonk, NY, USA) and NCSS2020 (NCSS Statistical Software, Kaysville, UT, USA). Figures were constructed with Prism (GraphPad Software, San Diego, CA, USA).

RESULTS

A total of 1001 children and adolescents were included in the final analysis (figure 1). The median (IQR) age was 5.6 (2.4–12.1)

Tuberculosis

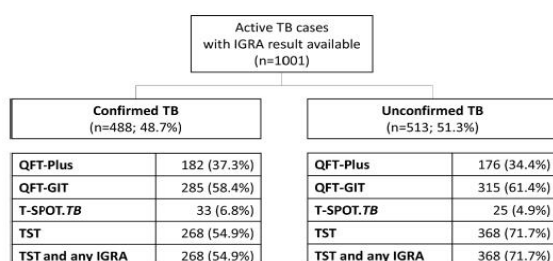


Figure 1 Flow chart summarising the composition of the study population and the type and number of tests performed in study participants with confirmed and unconfirmed TB. IGRA, interferon-gamma release assay; QFT-GIT, QuantiFERON-TB Gold in-Tube; QFT-Plus, QuantiFERON-TB Gold Plus; TB, tuberculosis; TST, tuberculin skin test.

years. Cases were contributed from participating healthcare centres located in 16 European countries: Spain (n=399), Italy (n=311), Germany (n=87), Turkey (n=32), United Kingdom (n=23), Latvia (n=22), Greece (n=21), Portugal (n=21), Bulgaria (n=20), Russia (n=19), Switzerland (n=15), Ukraine (n=15), Lithuania (n=7), Sweden (n=4), Croatia (n=3) and Austria (n=2). The demographic details of the study population are summarised in [table 1](#). Overall, 488 (48.8%) patients were classified as confirmed TB, and 513 (51.2%) as unconfirmed TB. Of the children with confirmed TB in whom all microbiological test results were available (n=435), 329 (75.6%) had positive MTB culture results and 300 (69.0%) had positive PCR results (n=194 both MTB culture-positive and PCR-positive). Approximately half (50.5%) had a history of TB contact (46.5% in the confirmed TB group vs 54.2% in the unconfirmed TB group, $p=0.009$). One hundred and thirty-six (13.6%) children had pre-existing comorbidities (online supplemental material table E1). Of those, eight had HIV coinfection, 20 were receiving immunosuppressive treatment, and 49 had other immune disorders. The most common disease manifestation was respiratory TB ([table 1](#)).

A QFT-Plus was performed in 358 cases (35.7%), QFT-GIT in 600 cases (59.9%), and T-SPOT.TB assay in 58 cases (5.8%). In 636 cases a quantitative TST result was available. A large proportion of children underwent more than one test: 375 (37.5%) QFT-GIT and TST, 224 (22.4%) QFT-Plus and TST, 29 (2.9%) T-SPOT.TB and TST, 5 (0.5%) QFT-GIT and T-SPOT.TB, 2 (0.2%) QFT-Plus and T-SPOT.TB and 6 (0.8%) QFT-GIT, T-SPOT.TB and TST.

Comparative performance of TST and IGRA assays

In the entire cohort, the overall sensitivity of the IGRAs and the TST at the 10 mm cut-off did not differ significantly: QFT-Plus 83.8% (95% CI 80.2% to 87.8%), QFT-GIT 85.5% (95% CI 82.7% to 88.3%), T-SPOT.TB 77.6% (95% CI 66.9% to 88.3%), TST10 83.3% (95% CI 80.4% to 86.2%). However, TST5 had significantly higher sensitivity than all three IGRAs in the entire cohort (89.9% (95% CI 87.6% to 92.3%)). [Table 2](#) shows a summary of the performance of each test in defined subgroups, based on demographics, disease focus and pre-existing comorbidities. There was no significant difference between the sensitivities of QFT-Plus and QFT-GIT in any of the subgroups analysed. While TST10 had lower sensitivity than QFT-Plus and QFT-GIT in patients with miliary TB and/or CNS TB, there was no significant difference observed between TST5 and both IGRAs in this subgroup. TST5 showed higher sensitivity than QFT-Plus in children aged 1–5 years, and higher sensitivity than all three IGRAs in patients with ‘other TB’.

Table 1 Baseline characteristics of the 1001 children included in the study

Age (years), median (IQR)	5.6 (2.4–12.1)
<1 year	101 (10.1)
1–5 years	426 (42.6)
6–10 years	170 (17.0)
>10 years	304 (30.4)
Gender with sex (male)*	537 (53.6)
Ethnic background	
White/Caucasian	505 (50.4)
Black/African American	91 (9.1)
Asian	70 (7.0)
Arab/Berber	52 (5.2)
Hispanic/Latin American	57 (5.7)
Mixed race	20 (2.0)
Unknown	206 (20.6)
Pre-existing comorbidities†	136 (13.6)
Underlying immunocompromise	77 (7.7)
HIV infection	8 (0.8)
Immunosuppressive treatment	20 (2.0)
Other immune disorder	49 (4.9)
BCG vaccination status	
BCG-vaccinated	235 (23.5)
BCG-unvaccinated	645 (64.4)
Unknown	121 (12.1)
Microbiologically-confirmed TB	488 (48.8)
Site of disease‡	
Respiratory	746 (74.5)
Miliary and/or CNS	88 (8.8)
Other focus §	166 (16.6)

Data shown are numbers and percentages unless stated otherwise.

*Gender unknown in n=1.

†For details regarding underlying medical conditions, see online supplemental material table E1.

‡Site of disease unknown in n=1.

§Other disease focus comprised: lymph node, osteoarticular, genitourinary, abdominal and pericardial TB.

BCG, Bacillus Calmette–Guérin; CNS, central nervous system; IQR, interquartile range; TB, tuberculosis.

All four tests showed lower sensitivity in the subgroups of children with miliary and/or CNS TB (QFT-Plus: 73.1%; QFT-GIT: 70.9%; T-SPOT.TB: 63.6%; TST10: 43.5%; TST5: 67.4%) and in immunocompromised children (QFT-Plus: 75.0%; QFT-GIT: 59.6%; T-SPOT.TB: 45.5%; TST10: 59.1%; TST5: 68.5%) compared with test sensitivity in the entire study population (QFT-Plus: 83.8%; QFT-GIT: 85.5%; T-SPOT.TB: 77.6%; TST10 83.3%; TST5: 89.9%).

Online supplemental material table E2 shows additional data in children with microbiologically-confirmed TB only, which are broadly consistent with the data shown in [table 2](#). None of the subgroup analyses showed a difference between the performance of QFT-Plus and QFT-GIT.

[Table 3](#), which summarises further comparisons of defined subgroups according to disease focus, shows that TST5, TST10 and QFT-GIT had significantly lower sensitivity in patients with miliary and/or CNS TB than in patients with pulmonary TB.

Tuberculosis

Table 2 Summary of the test performance of interferon-gamma release assays and the tuberculin skin test (at ≥ 10 mm and ≥ 5 mm cut-offs) in children with tuberculosis according to demographics, confirmation status and clinical characteristics

	No of patients with positive test result/no of patients tested Test sensitivity (95% CI)					QFT-Plus versus QFT-GIT *	TST10 vs			TST5 vs		
	QFT-Plus n=358	QFT-GIT n=600	T-SPOT.TB n=58	TST10 n=636	TST5 n=636		QFT-Plus*	QFT-GIT*	T-SPOT.TB*	QFT-Plus *	QFT-GIT *	T-SPOT.TB*
Entire cohort	300/358 83.8 (80.2 to 87.8)	513/600 85.5 (82.7 to 88.3)	45/58 77.6 (66.9 to 88.3)	530/636 83.3 (80.4 to 86.2)	572/636 89.9 (87.6 to 92.3)	0.47	0.84	0.29	0.26	0.004	0.017	0.004
Male	153/188 81.4 (76.3 to 87.3)	276/325 84.9 (81.0 to 88.8)	26/31 83.9 (70.9 to 96.8)	284/339 83.8 (79.9 to 87.7)	305/339 90.0 (86.8 to 93.2)	0.29	0.48	0.68	0.98	0.005	0.05	0.29
Female	147/170 86.5 (81.3 to 91.6)	236/274 86.1 (82.0 to 90.2)	19/27 70.4 (53.1 to 87.6)	245/296 82.8 (78.5 to 87.1)	266/296 89.9 (86.4 to 93.3)	0.92	0.29	0.27	0.11	0.26	0.17	0.003
Age <1 year	33/39 84.6 (73.3 to 95.9)	51/59 86.4 (77.7 to 95.2)	3/4 75.0 (32.6 to 100.0)	28/45 62.2 (48.1 to 76.4)	37/45 82.2 (71.1 to 93.4)	0.80	0.02	0.004	0.61	0.77	0.55	0.72
Age 1–5 years	114/141 80.9 (74.4 to 87.3)	230/272 84.6 (80.3 to 88.9)	15/17 88.2 (72.9 to 100.0)	235/285 82.5 (78.0 to 86.9)	255/285 89.5 (85.9 to 93.0)	0.34	0.68	0.5	0.54	0.014	0.08	0.87
Age 6–10 years	48/57 84.2 (74.7 to 93.7)	83/100 83.0 (75.6 to 90.4)	12/14 85.7 (67.4 to 100.0)	111/131 84.7 (78.6 to 90.9)	116/131 89.5 (83.1 to 94.0)	0.84	0.93	0.72	0.92	0.41	0.22	0.75
Age >10 years	105/121 86.8 (81.6 to 93.4)	149/169 88.2 (83.3 to 93.0)	15/23 65.2 (45.8 to 84.7)	156/175 89.1 (84.5 to 93.8)	164/175 93.7 (90.1 to 97.3)	0.72	0.53	0.77	0.0017	0.04	0.07	<0.0005
Confirmed TB	159/182 87.4 (81.6 to 91.8)	243/285 85.3 (80.6 to 89.2)	24/33 72.7 (54.5 to 86.7)	213/268 79.5 (74.1 to 84.1)	237/268 88.4 (84.0 to 92.0)	0.52	0.03	0.07	0.37	0.73	0.27	0.012
Unconfirmed TB	141/176 80.1 (73.4 to 85.7)	270/315 85.7 (81.3 to 89.4)	21/25 84.0 (64.0 to 95.5)	317/368 86.1 (82.2 to 89.5)	335/368 91.0 (87.6 to 93.7)	0.11	0.07	0.87	0.76	0.0003	0.03	0.24
Respiratory TB	230/268 85.8 (81.6 to 90.0)	386/445 86.7 (83.6 to 89.9)	35/41 85.4 (74.5 to 96.2)	413/487 84.8 (81.6 to 88.0)	441/487 90.6 (88.0 to 93.2)	0.73	0.70	0.40	0.92	0.05	0.06	0.28
Miliary and/or CNS TB	19/26 73.1 (56.0 to 90.1)	39/55 70.9 (58.9 to 82.9)	7/11 63.6 (35.2 to 92.1)	20/46 43.5 (29.2 to 57.8)	31/46 67.4 (53.8 to 80.9)	0.84	0.015	0.005	0.23	0.61	0.70	0.81
Other focus†	51/64 79.7 (71.3 to 90.6)	87/99 87.9 (81.4 to 94.3)	3/6 50.0 (10.0 to 90.0)	97/103 94.2 (89.7 to 98.7)	100/103 97.1 (93.8 to 100.0)	0.15	0.004	0.12	0.0001	0.0002	0.012	<0.0005
Pre-existing comorbidities	39/49 79.6 (68.3 to 90.9)	56/81 69.1 (59.1 to 79.2)	5/13 38.5 (12.0 to 64.9)	55/80 68.8 (58.6 to 78.9)	61/80 76.3 (66.9 to 85.6)	0.19	0.18	0.96	0.03	0.66	0.31	0.005
Immunocompromise (including HIV)	18/24 75.0 (57.7 to 92.3)	28/47 59.6 (45.5 to 73.6)	5/11 45.5 (16.0 to 74.9)	26/44 59.1 (44.6 to 73.6)	30/44 68.2 (54.4 to 81.9)	0.19	0.19	0.96	0.41	0.55	0.39	0.16

*P values based on two-sided Wald χ^2 tests with Bonferroni correction ($\alpha=0.017$) to account for multiple comparisons.

†Other disease focus comprised: lymph node, osteoarticular, genitourinary, abdominal and pericardial TB.

CNS, central nervous system; QFT-GIT, QuantiFERON-TB Gold in-Tube; QFT-Plus, QuantiFERON-TB Gold-Plus; TB, tuberculosis; TST, tuberculin skin test.

Online supplemental material table E3 shows additional data related to the performance of TSTs and IGRAs in different ethnic groups. In TST5 and TST10, the lowest sensitivity was observed in children with Hispanic/Latino background; additional analyses revealed that the sensitivity of TST5 and TST10 was significantly lower in this ethnic group than in Caucasian children ($p=0.0009$ and $p<0.0005$, respectively).

Online supplemental material table E4 shows a comparison of the retrospective and the prospective study arms. The additional analyses did not reveal any significant differences in the sensitivity of the investigated IGRAs (QFT-Plus, QFT-GIT and

T-SPOT.TB) between both arms. However, despite overlapping CIs, statistically the sensitivity of TST5 and TST10 was higher in the retrospective arm (TST5: 93.1% (95% CI 89.7% to 95.6%) vs 86.8% (95% CI 82.0% to 90.3%); TST10: 87.1% (95% CI 82.8% to 90.5%) vs 79.6% (95% CI 74.8% to 83.9%)).

Indeterminate IGRA results

Eleven (3.1%) of 358 patients who underwent QFT-Plus had an indeterminate test result, universally due to inadequate IFN- γ responses in the positive control. Twenty-one (3.5%) of 600

Table 3 Comparison of the test sensitivities of interferon-gamma release assays and the tuberculin skin test (at ≥ 5 mm and ≥ 10 mm cut-offs) stratified by disease focus

	Sensitivity (%)			Pairwise comparison (p value)		
	Respiratory TB	Miliary and/or CNS TB	Other focus*	Respiratory versus Miliary and/or CNS	Respiratory versus Other focus*	Miliary and/or CNS versus Other focus*
QFT-Plus	85.8	73.1	78.6	0.08	0.33	0.41
QFT-GIT	86.7	70.9	87.9	0.0019	0.76	0.008
T-SPOT.TB	85.4	63.6	50.0	0.10	0.04	<0.0005
TST5	90.6	67.4	97.1	<0.0005	0.03	<0.0005
TST10	84.8	43.5	94.2	<0.0005	0.011	<0.0005

P values were calculated with Wald χ^2 tests; according to Bonferroni correction, p values of <0.017 were considered statistically significant (highlighted in bold).

*Other disease focus comprised: lymph node, osteoarticular, genitourinary, abdominal and pericardial TB.

CNS, central nervous system; QFT-GIT, QuantiFERON-TB Gold in-Tube; QFT-Plus, QuantiFERON-TB Gold-Plus; TB, tuberculosis; TST, tuberculin skin test.

Tuberculosis

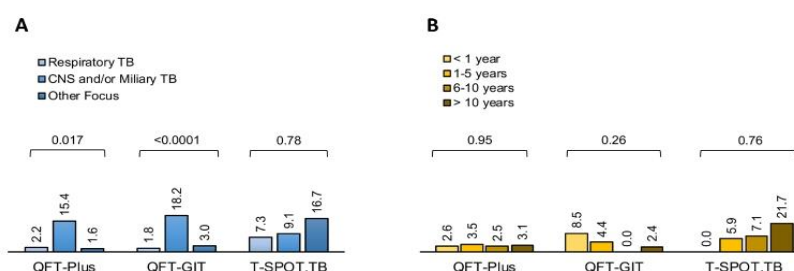


Figure 2 Proportion of indeterminate interferon-gamma release assay results according to assay and site of disease (A) and age group (B). Figures shown are percentages and p values calculated with Fisher's exact tests. CNS, central nervous system; QFT-GIT, QuantiFERON-TB Gold in-Tube; QFT-Plus, QuantiFERON-TB Gold Plus; TB, tuberculosis.

patients had an indeterminate QFT-GIT result, and 5 (8.6%) of 58 patients had an indeterminate T-SPOT.TB result.

The rate of indeterminate results was considerably higher in patients with miliary and/or CNS TB, compared with patients with respiratory TB and those with 'other focus', both for QFT-Plus ($p=0.017$) and QFT-GIT ($p<0.0001$), while this was not observed in T-SPOT.TB (figure 2A). Comparing indeterminate results across age groups (figure 2B), there was no statistically significant variation observed in any of the IGRAs. However, in children <1 year of age, the proportion of indeterminate results was substantially higher in QFT-GIT (5/59; 8.5%) than in QFT-Plus (1/39; 2.6%). Online supplemental material table E5 shows the proportions of indeterminate test results in confirmed and unconfirmed cases, as well as in immunocompromised and non-immunocompromised patients.

Negative IGRA and TST results

The proportion of negative test results did not vary substantially between the tests evaluated (table 4). A substantial proportion of patients with negative test results—varying between 36.1% and 75.0% between the tests—had microbiologically-confirmed TB, showing that those results were truly false-negative. Only a small proportion (5/47; 10.6%) of patients with negative QFT-Plus result were known to be immunocompromised, which may have accounted for the false-negative results in those particular patients (table 4).

Figure 3 shows comparisons of the proportion of negative test results according to site of disease and age for each test. TST5 showed significantly higher rates of negative results in children with miliary and/or CNS TB ($p<0.0001$), while the proportion of negative results in the IGRAs did not differ significantly between disease sites. Similarly, the proportion of negative test results did not vary significantly between age groups, although TST5 showed a trend of decreasing rates of negative results with increasing age ($p>0.05$). Online supplemental material table E5 shows that in all evaluated

tests negative results were more common in immunocompromised than in patients without immune compromise.

Quantitative TST and IGRA responses

Quantitative results were available for 272 QFT-GIT (45.3%) and for 281 QFT-Plus (78.5%) assays. Figure 4 shows quantitative TST results and background-corrected antigen responses in both QFT assays across different age groups. While no significant variation in the magnitude of IFN- γ responses was observed across the age groups in QFT-GIT, both QFT-Plus TB1 and TB2 responses varied significantly, with the highest median values being observed in children 1–5 years of age.

Figure 5 shows TST indurations and background-corrected antigen responses in QFT-GIT and QFT-Plus assays according to the site of disease. In all three tests, quantitative responses were lower in patients with miliary and/or CNS TB than in patients with pulmonary TB or 'other focus'.

Comparisons between IGRA and TST responses

Details regarding the correlation between categorical TST results (TST5 and TST10), and the test results of the three IGRAs are shown in online supplemental material table E6. Result concordance between TST and IGRA results was similar for all three IGRAs and overall higher with TST5, ranging from 87.3% (QFT-Plus) over 87.6% (QFT-GIT) to 88.6% (T-SPOT.TB). QFT-Plus had higher agreement with TST5 (Cohen's $k=0.462$) than QFT-GIT and T-SPOT.TB (Cohen's $k=0.343$ and $k=0.271$, respectively).

DISCUSSION

To our knowledge, this is the first multinational multicentre study investigating the diagnostic accuracy of QFT-Plus in comparison with other IGRA assays and the TST in children with TB disease, which was facilitated by the inclusion of a large number of

Table 4 Details regarding negative test results according to test in the entire study population

	No of patients undergoing test	No (%) with negative test result	No (%) with negative test result who had confirmed TB	No (%) with negative test result who were immunocompromised
QFT-Plus	358	47/358 (13.1)	17/47 (36.1)	5/47 (10.6)
QFT-GIT	600	66/600 (11.8)	25/66 (37.8)	18/66 (27.3)*
T-SPOT.TB	58	8/58 (13.7)	6/8 (75.0)	5/8 (62.5)*
TST5	636	64/636 (10.1)	31/64 (48.4)	14/64 (21.9)*
TST10	636	106/636 (16.6)	55/106 (51.8)	18/106 (17.0)*

*Includes two patients with human immunodeficiency virus infection.

QFT-GIT, QuantiFERON-TB Gold in-Tube; QFT-Plus, QuantiFERON-TB Gold Plus; TB, tuberculosis; TST, tuberculin skin test.

Tuberculosis

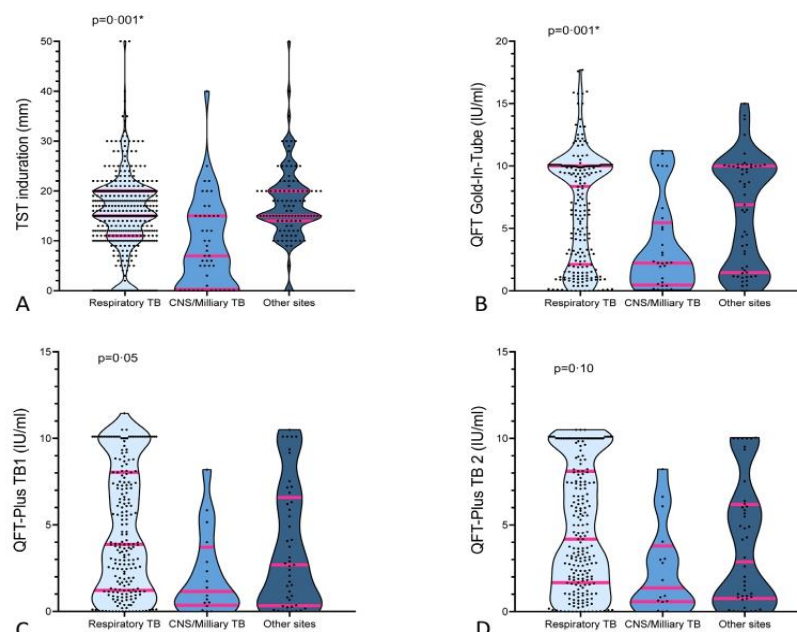


Figure 5 Violin plot of the quantitative results of the TST (A), the single antigen-stimulated sample of the QFT-GIT assay (B), and both antigen-stimulated samples of the QFT-Plus assay (TB1 (C) and TB2 (D)) according to disease site. All QFT-GIT and QFT-Plus data shown are background-corrected (ie, interferon-gamma concentration in the antigen-stimulated sample minus interferon-gamma concentration in the nil sample). The horizontal lines indicate median and IQRs; p values were calculated with Kruskal Wallis tests. CNS, central nervous system; QFT-GIT, QuantiFERON-TB Gold in-Tube; QFT-Plus, QuantiFERON-TB Gold Plus; TB, tuberculosis; TST, tuberculin skin test. *statistically significant.

paediatric study, which was conducted in Spain and included 158 paediatric patients with TB disease (46.8% microbiologically-confirmed), reported a substantially lower sensitivity estimate (82.9% (95% CI 77.0% to 88.8%)) than most adult studies.¹⁴ Those data are consistent with our sensitivity estimate of 83.8% (95% CI 80.2% to 87.8%) in a far larger paediatric study population recruited from 16 European countries. Another study from the same group, which also included children with suspected TB who ultimately had alternative diagnoses, found that QFT-Plus assays had a specificity, sensitivity, positive predictive value and negative predictive value of 91.5%, 87.3%, 86.4% and 91.2%, respectively.²⁶

We also found that there was a tendency for tests evaluated in our study to perform worse in patients with miliary and/or CNS TB, as well as in immunocompromised children. The former is concordant with the results of a recent study on TB meningitis in children from our network, which reported test sensitivities of 71.7% and 82.5% for QFT and T-SPOT.TB assays, respectively, highlighting that up to one in four children with CNS TB have false-negative IGRA results.²⁸ The poor performance of IGRAs in patients with disseminated TB has previously been reported in adult patients, with one study reporting the sensitivity of the QFT-GIT in adults with miliary TB to be as low as 68%.²⁹ The tendency for all four evaluated tests to perform worse in immunocompromised children is to be expected, considering that both the TST and IGRAs are based on the detection of functional immune responses directed against mycobacterial antigens. This observation also aligns with previous publications that have reported impaired IGRA performance in children with HIV infection and primary immunodeficiencies.¹⁸

Another key finding was that, overall, IGRAs performed similarly across all age groups, including in children <12 months of age, traditionally considered to be a group at higher risk of indeterminate assay results.^{11 16 18} However, on average quantitative IFN- γ responses in the QFT-Plus assay, both in TB1 and TB2 tubes, were lower in this particular age group than in the older age groups. Interestingly, in TST5 false-negative results were almost three times as common in children <12 months-of-age than in children >10 years-of-age (17.8% vs 6.3%), although this was not statistically significant. Also, the proportion of indeterminate QFT-GIT results in children <1 year was higher than in the older age groups, but this did not reach statistical significance.

Finally, our results highlight that there is an ongoing need to identify better immunological biomarkers (other than IFN- γ) that enable the development of improved immunodiagnostic TB tests for children.^{30–32} Promising biomarkers that have recently been described include mycobacteria-specific cytokine responses that have better performance characteristics than IFN- γ and may simultaneously facilitate the distinction between LTBI and TB disease, as well as polyfunctional MTB-specific T cells and MTB-specific antibody profiling.^{30 33–36}

Our study has several limitations. First, the study was predominantly retrospective, which may have introduced some selection bias. Second, only one IGRA was performed in the majority of cases, precluding head-to-head comparisons in individual patients. However, such direct comparisons are rarely performed in paediatric studies due to the large blood volume required for duplicate IGRAs. In some instances, the same individuals were included in

estimates of different test sensitivities, which limits our interpretation of independent effects between assays. Third, our study did not include children with LTBI or patients with suspected TB who were subsequently diagnosed with another condition, thereby precluding estimates regarding test specificity. Lastly, although our study included a large cohort of children from Western and Eastern Europe, children from the latter were less represented, mainly due to limited use of IGRAs in that region. However, a key strength of this study is the inclusion of a very large number of children with TB disease, enhancing the precision of our sensitivity estimates, and the involvement of a large number of study sites in different geographical locations, making the results generalisable to routine paediatric practice. However, additional data are needed in children with malnutrition and those living with HIV infection.

In conclusion, our study shows that IGRAs, including the last generation QFT-Plus assay, have suboptimal sensitivity in children and adolescents with TB disease, particularly those with mild and/or CNS TB and those with underlying immunocompromise, precluding their use as a rule-out test. QFT-Plus did not show greater sensitivity than previous generation IGRAs or the TST in our study population overall, as well as well-defined subgroups thereof. In light of our results, and considering the difficulty in obtaining microbiological confirmation of TB in children, the development of new immunodiagnostic tests that perform robustly across all age groups, ideally with the ability to distinguish between LTBI and TB disease, remains a key priority if the WHO TB elimination goals are to be achieved.

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Tuberculosis Disease in Immunocompromised Children and Adolescents: A Pediatric Tuberculosis Network European Trials Group Multicenter Case-control Study

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Background. In high-resource settings, the survival of children with immunocompromise (IC) has increased and immunosuppressive therapies are increasingly being used. This study aimed to determine the clinical characteristics, performance of diagnostic tools, and outcome of IC children with tuberculosis (TB) in Europe.

Methods. Multicenter, matched case-control study within the Pediatric Tuberculosis Network European Trials Group, capturing TB cases <18 years diagnosed 2000–2020.

Results. A total of 417 TB cases were included, comprising 139 children who are IC (human immunodeficiency virus, inborn errors of immunity, drug-induced immunosuppression, and other immunocompromising conditions) and 278 non-IC children as

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controls. Nonrespiratory TB was more frequent among cases than controls (32.4% vs 21.2%; $P = .013$). Patients with IC had an increased likelihood of presenting with severe disease (57.6% vs 38.5%; $P < .001$; odds ratio [95% confidence interval], 2.073 [1.37–3.13]). Children with IC had higher rates of false-negative tuberculin skin test (31.9% vs 6.0%; $P < .001$) and QuantiFERON-TB Gold assay (30.0% vs 7.3%; $P < .001$) results at diagnosis. Overall, the microbiological confirmation rate was similar in IC and non-IC cases (58.3% vs 49.3%; $P = .083$). Although the mortality in children with IC was $<1\%$, the rate of long-term sequelae was significantly higher than in non-IC cases (14.8% vs 6.1%; $P = .004$).

Conclusions. Children with IC and TB in Europe have increased rates of nonrespiratory TB, severe disease, and long-term sequelae. Immune-based TB tests have poor sensitivity in those children. Future research should focus on developing improved immunological TB tests that perform better in patients with IC, and determining the reasons for the increased risk of long-term sequelae, with the aim to design preventive management strategies.

Keywords. tuberculosis; immunodeficiency; immunosuppression; Europe; immune-based tests.

Tuberculosis (TB) remains a pressing global health concern, with children accounting for an estimated 10%–20% of the annual cases of TB. In 2021, the European Centre for Disease Prevention and Control recorded 6556 new cases in European children (<15 years), representing 4% of the total burden [1]. There are growing concerns in Europe that previous gains in TB control could be lost because of underdiagnosis during the coronavirus disease 2019 pandemic [2] and because of the increasing number of migrants to Europe from high-burden settings [3], including a recent surge in migration from Ukraine, one of the highest TB incidence countries in Europe [4].

The immune status plays a pivotal role in shaping the progression from *Mycobacterium tuberculosis* infection to TB [5, 6], and significantly influences disease severity [7]. Notably, in Europe, North America, and other high-income regions, there are growing populations of children with immunocompromise (IC) because of underlying chronic health conditions or immunosuppressive (IS) treatment [5, 7, 8]. Previous data suggest that children with Inborn Errors of Immunity (IEI), particularly those with T-cell defects, have an increased risk of infection and severe disease [7, 9–12], as do children with human immunodeficiency virus (HIV). During the past decade, the use of immunosuppressive drugs for rheumatological and autoimmune conditions, particularly of biological agents, has increased dramatically. Two types of biological agents, antibodies targeting tumor necrosis factor- α (TNF- α) and those targeting interleukin-1, have been shown to increase the risk of progression from *Mycobacterium tuberculosis* infection to TB several-fold compared with the general population, and therefore *M. tuberculosis* infection screening before treatment initiation is imperative [13–15].

Immunocompromised patients with TB tend to present with milder, nonspecific symptoms [16, 17], and more commonly have nonrespiratory TB [5]. In addition, there are in vitro and in vivo data showing that immune-based TB tests, including the tuberculin skin test (TST) and interferon-gamma release assays (IGRAs), perform worse in individuals with IC, which could result in delays in establishing a diagnosis of TB [6, 16, 18, 19]. Previous data also suggest that patients with IC are more likely to experience side effects related to TB

treatment [5, 6, 16], and that they are at substantially higher risk of developing long-term morbidity and fatal outcome [6, 16, 17, 20]. However, most of the existing data originate from small adult studies, often focused solely on HIV co-infection, and have typically been conducted at single centers in resource-constrained regions, where some diagnostic TB tests are not available in routine clinical practice [21, 22].

This study aimed to describe the clinical manifestations, disease severity, and treatment outcome of TB in children with IC in Europe, with comparisons to children with TB without known IC from the same setting. Additionally, the study aimed to assess the performance of immune-based and microbiological TB tests in both patient populations.

METHODS

We conducted a retrospective multicenter case-control study within the Pediatric Tuberculosis Network European Trials Group (ptbnet) [23], the largest research network dedicated to pediatric TB globally, comprising 416 members across 52 countries as of May 2023.

Inclusion Criteria

Patients younger than age 18 years at the time of their TB diagnosis, who had been diagnosed or treated at a European collaborating center between 2000 and 2020, were included in the study. Cases were defined as patients with IC with TB disease, whereas controls consisted of patients with TB disease without known IC (non-IC) (ie, no primary or secondary immunodeficiency and not receiving immunosuppressive treatment at the time of the TB diagnosis). For each IC case, 2 controls were matched in a 1:2 ratio. Matching criteria included sex and age, with age categories stratified as follows: <2 years, ≥ 2 –10 years, and ≥ 11 years.

Data Source

The data were sourced from the ptbnet database, a registry launched in 2017 to gather both retrospective and prospective data on children and adolescents <18 years who had TB contact, *M. tuberculosis* infection, or TB disease and received care at a participating healthcare center. The ptbnet database

is managed using REDCap electronic data capture tools [24], hosted at Gregorio Marañón Hospital in Madrid, Spain. This database captures comprehensive data covering demographics, clinical presentation, diagnostic tests, treatment modalities, and outcomes.

Definitions

We adhered to established consensus criteria for defining TB disease [25]. Patients with signs/symptoms of TB and a microbiological confirmation by culture and/or nucleic acid amplification tests (NAATs) were classified as confirmed TB. Unconfirmed TB was defined as a patient lacking microbiological confirmation but meeting at least 2 of the following criteria: (1) symptoms suggestive of TB, (2) chest radiograph consistent with TB, (3) known TB exposure or immunologic evidence of *M. tuberculosis* infection, and (4) response to TB treatment.

The anatomical site of TB disease was classified as respiratory TB, involving lung parenchyma, intrathoracic lymph nodes, larynx, trachea, bronchus, or pleura, or nonrespiratory TB, representing TB at any other site. Patients who had a respiratory disease focus and simultaneously had evidence of TB disease at other sites (eg, intra-abdominal lesions) were classified as having both respiratory TB and nonrespiratory TB. Miliary TB was defined as the acute hematogenous dissemination of miliary TB, evidenced by radiologic millet-like lung nodules, or by the isolation *M. tuberculosis* in blood/bone marrow.

Disease severity at diagnosis was classified as nonsevere TB in patients aged 3 months to <17 years, negative smear microscopy in respiratory samples, no rifampicin-/multidrug-resistance, and 1 of the following forms of disease: intrathoracic lymph node disease without airway obstruction, TB confined to a single lung lobe, noncavitary disease, nonmiliary pattern, uncomplicated pleural effusion, or uncomplicated peripheral lymphadenitis. Conversely, severe TB comprised patients not meeting those criteria according to established standards [26].

Immunocompromised was stratified into the following subgroups: (1) IEL, (2) HIV infection, (3) treatment with IS drugs (ie, conventional IS, biological agents or chemotherapy), (4) severe acute or chronic malnutrition (z score of <-3 or $<60\%$ weight for age or mid-upper-arm-circumference <115 mm), (5) other inborn diseases affecting immunity (eg, trisomy 21, sickle cell disease), and (6) other chronic diseases that impair immunological function (eg, renal failure, diabetes mellitus). Children with malnutrition and HIV infection were categorized as children with HIV.

Outcome data were stratified as cure, probable cure, or death [27]. Cure was defined as successful completion of prescribed treatment leading to the resolution of clinical symptoms, radiological improvement, and culture conversion. Probable cure consisted of the same criteria as cure, without documented culture conversion. Regarding death, we distinguished between patients who died because of TB and those who died from another

cause. Finally, we used poor outcome to categorize patients who died or developed chronic sequelae resulting from TB.

Statistical Analysis

A comprehensive analysis comparing clinical presentation, diagnostic performance, and outcomes between cases and controls was performed. Subgroup analyses focused on patients with IC, particularly children with HIV and those with drug-induced IC. Qualitative data were described using absolute frequencies and percentages, whereas quantitative data were summarized using mean, standard deviation, median, and interquartile range (IQR). The chi-square test was used for qualitative variables, and the Mann-Whitney U test for quantitative variables. For analyses related to the QuantiFERON-TB Gold assay (QFT; Qiagen, Hilden, Germany), results categorized as indeterminate or negative were collectively considered as “not positive.”

In the multivariate analyses, a backward stepwise binary logistic regression model was used with poor outcome as the dependent variable. Independent variables were those deemed clinically relevant and those with statistical significance on univariate analysis. All statistical tests used a 2-tailed approach and a significance level of 0.05, using IBM SPSS (version 27; IBM, Armonk, NY, USA).

Ethics

The study received approval from the Ethics Committee of each participating center. No identifiable information was collected. Written informed consent was obtained from the parents/guardians and/or of children aged ≥ 12 years, depending on local/national regulations.

RESULTS

A total of 417 participants were included in the study, comprising 139 cases with IC and 278 controls. Spanish centers contributed the largest number of participants ($n = 150$), followed by centers in Italy ($n = 59$), Germany ($n = 50$), Portugal ($n = 47$), Ukraine ($n = 37$), Russia ($n = 25$), United Kingdom ($n = 20$), Lithuania ($n = 17$), Sweden ($n = 4$), Bulgaria and Latvia ($n = 3$, each), and Austria ($n = 2$). Table 1 summarizes the demographic characteristics of the cases and the controls. Within the IC group, the largest subgroups were children with HIV and children receiving IS drugs (Table 2). Among children with HIV, the median CD4 count at the time of TB diagnosis was 292 cells/mm³ (IQR 71–493). For patients with drug-induced IS, the most commonly used medications were chemotherapy (16/37; 43.2%), corticosteroids (8/37; 21.6%), and anti-TNF-alpha agents (6/37; 16.2%).

Clinical Presentation and Disease Severity

With the exception of cough, all clinical symptoms/signs were more prevalent in IC than in non-IC patients (Table 3).

Table 1. Baseline Characteristics of the Study Population Comprising Immunocompromised (IC) Patients and Nonimmunocompromised (Non-IC) Controls

	Non-IC (n = 278)	IC (n = 139)	P Value
Age, y, median [IQR]	8.6 [4.1–14.4]	10.3 [6.2–13.5]	.362
Sex (female)	138 (49.6)	69 (49.6)	1.000
BMI, median [IQR]	16.6 [15.2–19.6]	15.5 [13.7–17.5]	<.001
BCG-vaccinated (n = 364)	99/250 (39.6)	58/114 (50.9)	.088
Migration status—immigrant (n = 405)	69/270 (25.6)	50/135 (37.0)	.017
WHO region of birth (n = 407)			
European	212/273 (77.7)	89/134 (66.4)	0.016
African	22/273 (8.1)	23/134 (17.2)	
Eastern Mediterranean	23/273 (8.4)	6/134 (4.5)	
Region of the Americas	11/273 (4.0)	11/134 (8.2)	
South-East Asia	4/273 (1.5)	5/134 (3.7)	
Western Pacific	1/273 (0.4)	0/134 (0.0)	
Ethnic background (n = 410)			
White Caucasian	154/276 (55.8)	66/134 (49.3)	.3216
Black/African American	39/276 (14.1)	28/134 (20.9)	
Latin American	25/276 (9.1)	16/134 (11.9)	
Asian	16/276 (5.8)	9/134 (6.7)	
Arab or Berber	19/276 (6.9)	6/134 (4.5)	
More than 1 ethnicity	7/276 (2.5)	4/134 (3.0)	
Other	16/276 (5.8)	5/134 (3.7)	
Other comorbidities (n = 359)			
Prematurity	3/226 (1.3)	6/133 (4.5)	.066
Cardiovascular	5/226 (2.2)	8/133 (6.0)	.066
Respiratory	7/226 (3.1)	7/133 (5.3)	.319
Neurological	6/226 (2.7)	12/133 (9.0)	.008
Gastrointestinal	0/226 (0.0)	11/133 (8.3)	<.001
Rheumatological	2/226 (0.9)	12/133 (9.0)	<.001
Nephrological	2/226 (0.9)	9/133 (6.8)	.002
Hematological disorders	3/226 (1.3)	28/133 (21.1)	<.001

Data shown are numbers and percentages unless stated otherwise. Data shown refer to the 417 patients included in the study, unless the number of patients with available data is specified in the denominator. Bold values represent the statistically significant results.

Abbreviations: BCG, Bacillus Calmette-Guérin; BMI, body mass index; IC, immunocompromised; IQR, interquartile range.

In both groups, the most commonly prevalent symptoms/signs were fever, cough, weight loss and asthenia. Similar proportions of IC and non-IC patients had respiratory TB (95.0% vs 91.4%). However, nonrespiratory disease was significantly more common in the IC than in the non-IC group (32.4% vs 21.2%). Also, the proportions of patients with miliary/disseminated TB, TB meningitis, and abdominal TB were significantly higher in the IC group than in the non-IC group (Table 3). Patients with IC also had an increased likelihood of presenting with severe disease (57.6% vs 38.5%; $P < .001$; odds ratio [95% confidence interval], 2.073 [1.37–3.13]).

Comparisons between non-IC patients and 4 IC subgroups (Supplementary Table 1), showed that miliary/disseminated TB was significantly more common in patients with IEL, patients with HIV, patients with drug-induced IC, and patients

Table 2. Subgroups of Immunocompromised Patients

	Number (%)
Inborn errors of immunity	5/139 (3.6%)
Chronic granulomatous disease	2
Mendelian susceptibility to mycobacterial disease	1
Hypogammaglobulinemia/CD16 deficiency	1
IFN-gamma/IL-17 release deficit	1
HIV infection ^a	41/139 (29.5%)
Immunosuppressive drugs ^b	37/139 (26.6%)
Chemotherapeutic agents	16
Steroids	8
Anti-TNF- α agents	6
Methotrexate	3
Azathioprine	2
Cyclosporine	1
Malnutrition ^c	25/139 (18.0%)
Other inborn diseases affecting immunity ^d	20/139 (14.4%)
Trisomy 21	10
Sickle cell disease	10
Other chronic conditions ^e	19/139 (13.7%)

Data shown are numbers and percentages unless stated otherwise.

Abbreviations: IFN, interferon; HIV, human immunodeficiency virus; IL, interleukin; TNF, tumor necrosis factor.

^aThree children with HIV were also malnourished.

^bAmong children receiving immunosuppressive drugs, 3 received >1 drug; the specific drug was unknown in 2.

^cEleven children with malnutrition had other conditions (HIV n = 3, chronic conditions n = 7, inherited disorders n = 1).

^dFive children with other inborn diseases affecting immunity had other conditions (drug-induced IC n = 4, malnutrition n = 1).

^eChronic conditions: diabetes mellitus (n = 10), chronic renal failure (n = 3), Duchenne (n = 1), severe cerebral palsy (n = 1), idiopathic CD4 lymphocytopenia (n = 1), solid organ tumor (n = 1), rheumatologic conditions (n = 2).

with malnutrition than in non-IC patients. All 4 IC subgroups also had higher proportions of severe TB disease than the group of non-IC patients.

Diagnostic Test Results

Chest radiograph findings were documented in 412 (98.8%) children and 272 (65.2%) underwent a computed tomography scan. Similar proportions of patients with IC and non-IC had abnormal findings on chest radiograph and/or chest computed tomography (Table 4). There were no significant differences in the radiological abnormalities identified between both groups, with the exception of miliary changes, which were significantly more common in the IC group.

False-negative TST results (<5 mm) were significantly more common in patients with IC than in those without (31.9% vs 6.0%; $P < .001$; Table 4 and Figure 1). Children with IC also had smaller TST indurations on average than children without IC (12 mm vs 15 mm; $P < .001$). Similarly, the proportion of false-negative QFT results was far higher in the IC than in the non-IC group (30.0% vs 7.3%; $P < .001$); in contrast, the proportion of indeterminate QFT results did not differ significantly between both groups.

Table 3. Reason for TB Screening, Clinical Characteristics at Presentation, Site of Disease, Disease Severity, and Outcome of Immunocompromised (IC) and Nonimmunocompromised (Non-IC) Study Participants

	Non-IC (n = 278) n (%)	IC (n = 139) n (%)	P Value n (%)
Reason for TB screening (n = 406)			
Contact tracing	109/271 (40.2)	27/135 (20.0)	.002
TB signs/symptoms	142/271 (52.4)	81/135 (60.0)	.254
Migrant screening	20/271 (7.4)	2/135 (1.5)	.169
IC screening	0 (0)	25/135 (18.5)	<.001
Clinical symptoms at presentation (n = 413)	192/277 (69.3)	120/136 (88.2)	<.001
Fever (n = 411)	109 (39.2)	72 (51.8)	<.001
Cough (n = 398)	120 (44.3)	60 (47.2)	.580
Respiratory distress (n = 387)	20 (7.7)	25 (19.7)	<.001
Weight loss (n = 389)	76 (29.0)	60 (47.2)	<.001
Asthenia (n = 391)	80 (30.4)	61 (47.7)	<.001
Vomiting (n = 307)	7 (3.4)	11 (11.1)	.007
Other GI symptoms (n = 339)	21 (9.1)	18 (16.5)	.047
Hepatomegaly (n = 386)	3 (1.1)	23 (16.5)	<.001
Splenomegaly (n = 387)	7 (2.5)	16 (11.5)	<.001
Headache (n = 381)	6 (2.3)	10 (8.2)	.008
Peripheral lymphadenopathy (n = 385)	28 (10.7)	24 (19.5)	.018
Site of TB (n = 416)			
Respiratory disease	254 (91.4)	132 (95.0)	.184
Non-respiratory disease ^a	59 (21.2)	45 (32.4)	.013
Miliary/disseminated TB	13 (4.7%)	33 (23.7%)	<.001
TB meningitis	6 (2.2%)	17 (12.2%)	<.001
Abdominal TB	10 (3.6%)	18 (12.9%)	<.001
Peripheral lymph node TB	25 (9%)	14 (10.1%)	.721
Osteoarticular TB	18 (6.5%)	11 (7.9%)	.586
Genitourinary TB	1 (0.4%)	1 (0.7%)	.616
Disease severity and outcome			
Severe TB at diagnosis	107 (38.5)	80 (57.6)	<.001
Poor outcome (n = 392)	16/263 (6.1)	20/129 (15.6)	.002

Data shown are numbers and percentages unless stated otherwise. Data shown refer to the 417 patients included in the study unless the number of patients with available data is specified in parentheses. Bold values represent the statistically significant results.

Abbreviations: CNS, central nervous system; GI, gastrointestinal; IC, immunocompromised; IQR, interquartile range; LN, lymph node; TB, tuberculosis.

^a73 participants with nonrespiratory disease also had respiratory disease.

Subgroup analyses (Supplementary Table 1) showed that the rate of false-negative TST results (<5 mm), was significantly higher in patients with HIV, drug-induced IC, or malnutrition than in non-IC patients. Also, the rate of false-negative QFT results was substantially higher in all 4 IC subgroups than in the non-IC group. The highest proportion of indeterminate QFT results (16.7%) was observed in the subgroup of patients with malnutrition.

TST and QFT agreement was assessed in 163 children, comprising 30 IC and 133 non-IC patients. Among non-IC patients, there was a positive agreement (TST+/QFT+) in 87.1%, negative agreement (TST-/QFT-) in 3.8%, TST+/QFT- discordance in 7.6%, and TST-/QFT+ discordance in 2.3%. In contrast, the

Table 4. Immune-based, Microbiological, and Radiological Tests in the Study Population

	Non-IC (n = 278)	IC (n = 139)	P Value
TST; median [IQR] ^a	15 [12–20]	12 [5–17]	<.001
TST <5 mm	11/183 (6.0)	22/69 (31.9)	<.001
TST ≥5 mm	172/183 (94.0)	47/69 (68.1)	<.001
Categorical QFT result			
Negative	14/191 (7.3)	21/70 (30.0)	<.001
Indeterminate	9/191 (4.7)	2/70 (2.9)	<.732
Positive	168/191 (88.0)	47/70 (67.1)	<.001
TB microbiological confirmation			
Confirmed TB	137 (49.3)	81 (58.3)	.083
Culture-positive ^b	116/252 (46.0)	70/129 (54.3)	.128
NAAT-positive ^b	96/220 (43.6)	58/96 (60.4)	.006
Chest x-ray findings	222/274 (81.0)	105/138 (76.1)	.243
Hilar lymphadenopathy	105/215 (48.8)	40/103 (38.8)	.094
Parenchymal opacities	142/220 (65.0)	60/101 (59.4)	.323
Calcifications	10/178 (5.6)	4/82 (4.9)	.790
Cavities	27/211 (12.8)	8/100 (8.0)	.211
Miliary pattern	8/210 (3.8)	25/102 (24.5)	<.001
Chest CT findings	167/172 (97.1)	97/100 (97.0)	.965
Hilar lymphadenopathy	106/163 (65.0)	55/94 (58.5)	.298
Parenchymal opacities	111/164 (67.7)	62/94 (66.0)	.777
Calcifications	28/143 (19.6)	13/76 (17.1)	.655
Cavities	30/159 (18.9)	12/88 (13.6)	.295
Miliary pattern	10/158 (6.3)	26/90 (28.9)	<.001

Data shown are numbers and percentages unless stated otherwise. Data shown refer to the 417 patients included in the study unless the number of patients with available data is specified in denominators. Bold values represent the statistically significant results.

Abbreviations: CT, computed tomography; IC, immunocompromised; NAAT, nucleic acid amplification test; QFT, QuantiFERON-TB Gold; TST, tuberculin skin test.

^aTST induration was not recorded in 83 participants.

^bBoth culture and NAAT were positive in 119 participants.

IC group showed a positive agreement in only 53.3% of the patients, negative agreement in 23.3%, TST+/QFT- discordance in 13.3%, and TST-/QFT+ discordance in 10.0%.

Children with IC exhibited a higher proportion of microbiologically confirmed TB compared with non-IC patients (58.3% vs 49.3%; $P = .083$; Table 4), with a significantly higher proportion of patients having positive NAATs results in the former group (60.4% vs 43.6%; $P = .006$). Children with HIV had a lower microbiological confirmation rate than children in the other IC subgroups (43.9% vs 64.3%, $P = .026$) (Supplementary Table 2).

There were no statistically significant differences in the microbiological yield of different sample types between IC and non-IC patients: gastric aspirates (43.8% vs 43.8%, $P = .990$), spontaneous sputum (50.0% vs 68.8%, $P = .082$), induced sputum (33.3% vs 36.8%, $P = .788$), and fine-needle aspiration (72.7% vs 55.0%, $P = .332$).

Treatment and Outcome

On average, patients with IC received longer courses of anti-TB treatment compared with non-IC patients (8.5 months [IQR:

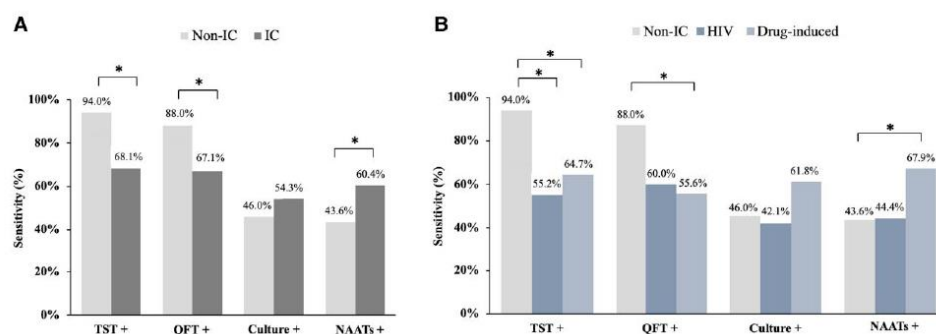


Figure 1. Sensitivity of immunological and microbiological tests according to immune status. Bar chart showing sensitivity of immunological and microbiological tests in nonimmunocompromised (non-IC) and immunocompromised (IC) patients (A), and non-IC patients, patients with HIV, and patients with drug-induced immunocompromise (B). Comparisons were made with chi-square tests; statistically significant differences ($P < .05$) are indicated by *. Abbreviations: IC, immunocompromised; HIV, human immunodeficiency virus; NAATs, nucleic acid amplification tests; QFT, QuantiFERON-TB Gold; TST, tuberculin skin test.

6–12] vs 6.3 months [IQR: 6–9], $P < .001$). Drug-related adverse events occurred in 82 children (32.4% [36/111] of IC and 24.2% [46/190] of non-IC patients, $P = .207$), with the most frequent events being elevation of liver enzymes (36.4%) and gastrointestinal symptoms (24.2%). Adherence to treatment was good overall, with no significant difference between IC and non-IC patients (94.7% vs 93.7%, $P = .680$); almost all children completed the prescribed course of treatment, except for 8 cases (4 IC and 4 non-IC patients).

Outcome data for 392 children revealed a probable cure rate of 72.7% in IC versus 80.7% in non-IC patients, and cure rates of 26.6% and 19.3%, respectively. The IC group had a higher rate of poor outcome (ie, death or chronic sequelae) compared with the non-IC group (15.6% vs 6.1%, $P = .002$). There was only 1 reported death—a 10-year-old girl with severe malnutrition and epilepsy who died of miliary TB 1 month after treatment initiation. Long-term sequelae were significantly more common in IC than in non-IC patients (14.8% vs 6.1%; $P = .004$), with the most common types comprising respiratory (7.8% vs 1.4%; $P = .003$), osteo-articular (3.9% vs 2.3%; $P = .358$), and neurologic sequelae (2.3% vs 0.8%; $P = .335$).

In the multivariate analyses related to outcome (Table 5), IEI and malnutrition were identified as IC conditions associated with poor outcome (IEI: odds ratio, 9.54 [1.50–60.34], $P = .017$; malnutrition: odds ratio, 3.87 [1.39–10.78], $P = .009$). Other factors associated with poor outcome were non-European origin (odds ratio, 3.3 [1.64–6.64], $P < .001$), presence of respiratory distress at presentation (odds ratio, 4.24 [1.67–7.31], $P = .002$) and nonrespiratory TB (odds ratio, 8.71 [3.85–19.73], $P < .001$). Finally, having a negative QFT result at presentation was also associated with poor outcome (odds ratio, 9.41 [2.27–38.99]; $P = .002$).

DISCUSSION

This European case-control study currently represents the largest multicenter study focusing on TB disease in children with IC in middle- to high-income countries, and provides valuable contemporary data related to this high-risk patient group. HIV infection and drug-induced IC were the predominant conditions in this cohort, the latter reflecting the increasing prevalence of autoimmune-mediated diseases in high-resource countries [28].

In concordance with previous studies in people with HIV and studies focusing on TB in patients with drug-induced IS, we found that children with IC had significantly higher rates of nonrespiratory TB and disseminated/miliary TB [5, 16]. Further analyses also revealed that all IC subgroups—children with IEI, children with HIV, children with drug-induced IS, children with malnutrition—were at increased risk of severe TB disease compared with non-IC controls. Both observations can be explained by the reduced ability to contain *M. tuberculosis* in patients with IC, which requires a complex functioning interaction between innate immune cells and T cells [29].

Our study also highlights the limitations of immune-based TB tests in children with IC. Overall, close to one third of the patients had false-negative TST (at the 5-mm cutoff) and/or QFT results. In contrast to some previous studies, in our cohort, the sensitivity of the QFT assay was not greater than that of the TST [30, 31]. Consequently, although in children with IC presenting with features consistent with TB, a positive TST or QFT result can be useful to support a putative diagnosis of TB; negative results should not distract from further investigations, including imaging and microbiological tests. Considering that TST/IGRA discordance is not uncommon in children with TB disease, especially in those with IC, we believe that both tests should be done in parallel to increase the

Table 5. Multivariate Analyses of Factors Potentially Associated With Poor Outcome

	Good Outcome (n = 356)	Poor Outcome (n = 36)	P Value	OR (95% CI)	P Value
IC disorder					
Inborn errors of immunity	3/5 (60.0)	2/5 (40.0)	.069	9.54 (1.50–60.34)	P = .017
HIV infection	32/37 (86.5)	5/37 (13.5)	.338	2.27 (.78–6.62)	P = .131
Immunosuppressive drugs	30/33 (90.9)	3/33 (9.1)	1.000	0.61 (.13–2.75)	P = .520
Malnutrition	17/23 (73.9)	6/23 (26.1)	.004	3.87 (1.39–10.73)	P = .009
Other inborn diseases	15/19 (78.9)	4/19 (21.1)	.085	1.72 (.40–7.39)	P = .461
Other chronic conditions	17/18 (94.4)	1/18 (5.6)	1.000	2.49 (.80–7.79)	P = .115
Non-European ^a	82/100 (82.0)	18/100 (18.0)	<.001	3.30 (1.64–6.64)	P < .001
Clinical presentation					
Respiratory distress	32/353 (9.0)	10/36 (27.8)	.002	4.24 (1.67–10.77)	P = .002
Nonrespiratory TB	69/355 (19.4)	26/36 (72.2)	<.001	8.71 (3.85–19.73)	P < .001
Diagnostic tests					
Negative TST	26/220 (11.8)	5/17 (29.4)	.038	0.60 (.67–7.31)	P = .765
Negative QFT result	34/229 (14.8)	8/21 (38.1)	.006	9.41 (2.27–38.99)	P = .002
Culture confirmation	155/328 (47.3)	19/35 (54.3)	.429	0.69 (.10–4.81)	P = .716
NAAT confirmation	126/275 (45.8)	19/28 (67.9)	.026	1.22 (.13–10.90)	P = .857

Data shown are numbers and percentages unless stated otherwise. Bold values represent the statistically significant results.

Abbreviations: CI, confidence interval; IC, immunocompromise; NAAT, nucleic acid amplification tests; OR, odds ratio; QFT, QuantiFERON-TB Gold; TB, tuberculosis; TST, Tuberculin skin test.

^aRegion of birth.

diagnostic yield, as also recommend by clinical practice guidelines [32]. We found that overall microbiological confirmation, by both culture and NAATs, is more commonly achieved in children with IC than those without IC. However, subgroup analyses revealed that children with HIV had lower confirmation rates than children with other forms of IC and non-IC children. This finding is in line with previous studies in adults with HIV, which also reported low yields of microbiological investigations in this particular patient group [22, 33].

Previous studies from low-resource settings have highlighted the increased risk of long-term sequelae and fatal outcome in patients with IC, including in people with HIV [34, 35]. In our cohort, only 1 child with IC died, equating to a mortality rate below 1%. This observation is likely explained by the fact that all participating units had access to the full range of radiological and microbiological diagnostics, thereby enabling timely diagnoses, as well as high-level intensive care support. However, despite the availability of those facilities, a substantial proportion (15.6%) of children with IC had a poor outcome. Long-term sequelae resulting from TB were significantly more common in children with IC than in those without IC (14.8% vs 6.1%). Multivariate analyses revealed several risk factors for poor outcome in children with IC, including underlying IEI or malnutrition, presence of respiratory distress at presentation, presence of nonrespiratory TB, and a negative QFT result at presentation. The latter observation is intriguing and may reflect reduced numbers of *M. tuberculosis*-specific T cells in the peripheral circulation or alternatively T-cell energy [36].

Our study has certain limitations. First, because of its retrospective nature some data are missing and there is a risk of

selection bias. Also, given the limited number of patients with TB matched by sex and age in some countries, it was not always possible to also match cases and controls according to their country of origin. The diverse management practices in the different participating units may limit the generalizability of our observations to low-income countries. In addition, there is limited access to IGRAs in some participating centers in middle-income countries. Furthermore, not all included patients were microbiologically confirmed, but this is a common issue in pediatric TB studies, in which confirmation rates are typically far lower than in adult studies. Additionally, of the 13 children with HIV who had radiological findings consistent with miliary TB, only 5 had microbiologically confirmed TB; in the remaining 8 the possibility of lymphoid interstitial pneumonitis cannot be excluded with certainty. Last, the relatively small number of children with IEI included in this cohort restricted our ability to conduct robust comparative analysis within this subgroup.

In summary, our study of IC children with TB in Europe found increased rates of nonrespiratory TB, severe disease, and long-term sequelae within this group. Immune-based TB tests had poor sensitivity, with approximately one third of IC children having negative TST and/or IGRA results. Overall, the sensitivity of microbiological tests was similar in children with IC and those without IC, with the exception of children with HIV in whom microbiological confirmation of TB disease was significantly lower. Future research should focus on the development of improved immunological TB tests that have better performance characteristics than existing tests in children with IC. Furthermore, more research is needed to

explore the reasons for the increased rate of long-term sequelae in children with IC, ultimately with the aim to design preventive management strategies to avert adverse outcomes.

Supplementary Data

Supplementary materials are available at *Clinical Infectious Diseases* online. Consisting of data provided by the authors to benefit the reader, the posted materials are not copyedited and are the sole responsibility of the authors, so questions or comments should be addressed to the corresponding author.

Notes

Author contributions. P. R. M., M. T., T. S., L. F. A., and B. S. G. conceptualized and designed the study; P. R. M., L. F. A., and A. H. B. collected data; B. S. G. coordinated and supervised data collection; P. R. M. and L. F. A. drafted the initial manuscript; M. D. A., B. S. G., and P. R. M. carried out the initial analyses. All authors critically reviewed the data and revised the manuscript. All authors approved the final submitted manuscript and agreed to be accountable for all aspects of the work.

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Potential conflicts of interest. B. S. G. and M. T. have received support from Cepheid for a project on molecular tuberculosis diagnostics unrelated to the study reported here. M. T. has received QuantiFERON-TB assays at reduced pricing or free of charge for tuberculosis diagnostics projects from Cellestis/Qiagen in the past and has received support for conference attendance from Cepheid. The manufacturers had no influence on the study design, data collection, analysis or interpretation, writing of the manuscript, or the decision to submit the data for publication. All other authors report no potential conflicts.

All authors have submitted the ICMJE Form for Disclosure of Potential Conflicts of Interest. Conflicts that the editors consider relevant to the content of the manuscript have been disclosed.

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CHAPTER 4

Immunological and metabolomic profile evaluation of pediatric TB spectrum

1. Introduction

Despite significant progress in TB control over recent decades, the disease remains a major global health challenge and continues to be one of the leading causes of mortality in children worldwide (1). The COVID-19 pandemic further compounded these challenges by disrupting TB screening programs and reducing opportunities for the timely detection of both active TB and LTBI, raising serious concerns about a resurgence of cases in the near future (2).

Early identification and treatment of *Mycobacterium tuberculosis* infection are fundamental to preventing progression to active disease and are critical to achieving international TB elimination goals (3). Current immunological tools, such as the TST and IGRAs, are widely used for this purpose (4). These assays detect *Mtb*-specific immune responses but cannot distinguish LTBI from active TB, nor do they provide prognostic information (4). Moreover, there is evidence suggesting that IGRAs perform worse in children compared to adults, with lower sensitivity and a higher rate of indeterminate results (5,6). Recent evaluations have also shown that, contrary to expectations, QFT-Plus did not perform better than earlier IGRAs or the TST in terms of sensitivity (7,8). Children are at higher risk than adults of developing active disease and of progressing to severe and disseminated forms, yet establishing a definitive diagnosis in this vulnerable population remains particularly challenging (9,10). Several factors hinder the differentiation between LTBI and active TB: clinical signs and symptoms are often non-specific, radiological findings may lack distinct features, and microbiological confirmation is hampered by the paucibacillary nature of childhood TB as well as the limited ability of children to produce sputum samples. Even with the introduction of sensitive molecular techniques, diagnostic confirmation in children remains difficult (11,12).

Adding further complexity to the scenario, recent models of *Mtb* infection have reconsidered the traditional dichotomy between LTBI and active TB disease in favor of a continuous spectrum of infection. This spectrum encompasses stages ranging from

exposure and latent infection to incipient, subclinical, and active TB, reflecting progressive degrees of bacterial replication, metabolic activity, and host immune response. The immunological mechanisms underlying TB progression, persistence, and reactivation remain poorly understood (13). In this context, identifying biomarkers capable of distinguishing, or even predicting, the different stages of infection, particularly incipient and subclinical TB, could give opportunities for the early recognition of preclinical conditions and guide interventions to prevent progression and transmission. Multiple efforts have been undertaken to design novel immunological assays with improved accuracy for TB stratification, either by employing alternative *Mtb* antigens to activate peripheral T cells or by measuring cytokines and chemokines other than IFN- γ after ex vivo stimulation (14,15).

One promising candidate is the mycobacterial heparin-binding hemagglutinin adhesin (HBHA), a latency-associated antigen. HBHA-induced IFN- γ release assays have shown encouraging results in adults, with HBHA-specific T-cell responses associated with TB containment and the potential to differentiate LTBI from active TB. These findings, however, have largely been limited to adult populations, and pediatric data remain scarce (16,17,18). Given these gaps, combining the QFT-Plus assay with an HBHA-based IGRA could represent a valuable approach to refine the stratification of TB infection in children and provide insights into treatment monitoring.

Metabolomics, defined as the comprehensive qualitative and quantitative analysis of small-molecule metabolites within a biological system, has recently gained attention as a tool in biomedical research. By capturing dynamic changes in metabolic pathways, metabolomic profiling may provide insights into host–pathogen interactions and is increasingly being applied to infectious diseases (19). In the context of TB infection, disturbances occur in host energy, lipid, and amino acid metabolism, which may be reflected in distinct metabolic signatures detectable in blood, urine, or other accessible samples. *Mtb* itself can exist in an actively replicating state, characterized by predominantly aerobic metabolism, or in a dormant, non-replicating persistent state with anaerobic or microaerophilic metabolism. These bacterial populations exhibit distinct transcriptional and proteomic profiles that influence their interaction with the host and their susceptibility to immune-mediated control (20).

Although encouraging studies have demonstrated the applicability of metabolomics in tuberculosis, evidence, particularly in children, remains limited.

Integrating infection-induced metabolic profiling with novel immunological assays may enable the identification of new biomarkers of TB, ideally capable of distinguishing latent infection from active disease, potentially allowing a finer distinction between different disease stages, and providing reliable tools for monitoring treatment response.

This study was carried out as part of my doctoral research and was awarded funding through the PRIN (*Progetti di Ricerca di Interesse Nazionale*) program, as the winning project of call R0000972 (30/04/2023, prot. 2022K5AXKB).

This prospective study aims to evaluate the utility of a combined approach—integrating an HBHA-based IGRA with QFT-Plus—in distinguishing active TB from LTBI in children, and possibly different stages of the TB spectrum, and in monitoring immunological changes during therapy.

In this context, metabolomic profiling, to be performed using an untargeted approach, may provide a valuable complement to immunological assays, offering additional means to differentiate infection stages and to identify markers predictive of treatment response and disease progression.

2. Methods

2.1 Study design and setting

We conducted a prospective two-center study on children aged below 18 years and referred for clinical suspect or TB screening at the Pediatric Infectious Diseases Unit of the University Federico II, in Naples and at Fondazione Policlinico Universitario A. Gemelli University Hospital, in Rome between September 28th, 2023 and September 1st, 2025. Both centers are identified as pediatric referral centers for the management of TB infection in the respective regions, Campania and Lazio, regions with more than 5 million inhabitants and about 1 million children each, and with a population density among the highest in Italy (24,25).

Children whose caregivers refused to provide informed consent and children bearing potential confounding factors that may hamper an appropriate evaluation of the immunological and metabolomic profile (AIDS or HIV infection, primary and secondary immunodeficiencies, onco-hematological disease undergoing chemotherapy treatment, reactivation of TB infection after previous treatment) were excluded from the study.

2.2 Clinical evaluation and TB management

All enrolled children underwent clinical evaluation, TST, and IGRA test (QFT-Plus). In cases with results suggestive of TB infection, chest X-ray (CXR) and microbiological investigations were conducted, chest computed tomography (CT) was performed as needed to refine the diagnosis. Microbiological assessment included smear microscopy for acid-fast bacilli (Ziehl–Neelsen staining), culture on solid (Löwenstein–Jensen) and liquid (BACTEC MGIT 960) media, and molecular testing with the Xpert MTB/RIF Ultra assay (Cepheid) for detection of *Mycobacterium TB* complex and rifampicin resistance. Based on clinical, radiological and diagnostic work-up, patients were classified into one of the following categories (26):

- **Active TB**, disease, defined as infection with *Mycobacterium tuberculosis*, *Mtb* (scored positive to QTF-Plus and-or TST) and radiological and/or clinical evidence of active disease;
- **LTBI**, defined as infection with *Mtb* in the absence of clinical or radiological evidence of disease;
- **Exposed** children, defined as subjects with a history of recent contact (<3 months) with a contagious TB case, without evidence of infection, and with normal clinical and radiological findings;
- **“non TB”**, defined as healthy children who were screened for LTBI and showed no evidence of infection or children admitted for presumed TB who were eventually diagnosed with any infectious disease other than active TB or LTBI.

Following diagnosis, children were managed according to standardized international recommendations (27,28). Antitubercular therapy was initiated under the following conditions: (i) isoniazid for 2 months in exposed children under 5 years of age until repetition of TST and/or IGRA testing to rule out infection conversion; (ii) LTBI treatment with isoniazid for 6 months; and (iii) treatment of active TB consisted of a 2-months intensive phase with isoniazid, rifampicin, pyrazinamide, and, when indicated, ethambutol, followed by a 4-months continuation phase with isoniazid and rifampicin, regardless of severity and in the absence of complications, or by a 10-months continuation phase in meningeal and osteoarticular TB.

In patients with non-severe forms of active TB, short-course therapy was deliberately not employed to avoid introducing confounding factors.

2.3 Definitions of active TB

Children initially diagnosed with active TB were classified according to established consensus criteria (4,28,29):

- Confirmed TB, microbiological confirmation of *Mtb* infection by culture and/or molecular testing, or unconfirmed TB;

- Pulmonary TB, disease involving the lung parenchyma (including the tracheobronchial tree or miliary dissemination or intrathoracic lymphadenopathy (mediastinal and/or hilar) without radiographic abnormalities in the lungs;
- Extrapulmonary TB, disease affecting organs or tissues outside the lung parenchyma (e.g. peripheral lymph nodes, pleura, meninges, bone, genitourinary tract, peritoneum) in the absence of pulmonary lesions. For the purpose of this study cases with both pulmonary and extrapulmonary involvement were classified considering the most severe clinical manifestation (e.g. a child with pulmonary TB and meningitis is classified as meningitis);
- Symptomatic TB, at least one of the following: persistent cough > 2 weeks, weight loss or failure to grow, prolonged fever > 2 weeks, night sweats, persistent non-tender lymphadenopathy, symptoms specific to extrapulmonary sites (e.g., bone pain, neck stiffness, neurological symptoms, persistent ascites), or non-symptomatic TB;
- Severe TB, at least one of the following:
 - i) pulmonary disease affecting more than one lobe of the lungs or cavitary or with a miliary pattern
 - ii) extrapulmonary TB except to peripheral lymph node TB, intrathoracic lymph node TB without airway obstruction, uncomplicated TB pleural effusion;
 - iii) symptoms that require hospitalization.

At the end of follow-up, children were classified according to the following outcomes: (1) cure without sequelae, (2) cure following need for prolonged treatment or modification of drug regimen, (3) cure with sequelae, (4) treatment failure/relapse, (5) death, and (6) loss to follow-up.

2.4 Data and biological sample collection

Trained personnel at each center prospectively collected in a Clinical Record Form (CRF) epidemiological data, demographic characteristics, history of exposure, comorbidities,

initial symptoms, clinical signs, disease localization, microbiological and radiological findings, treatments and disease courses during hospitalization and follow-up.

In patients diagnosed with LTBI or active TB, experimental samples for immunological and metabolomic analysis (serum, saliva, urine) were collected at the time of diagnosis (T0), after 2 months of treatment (T1), after 4 months of treatment (T2), at 6 months, corresponding to the end of treatment for LTBI and pulmonary TB (T3) and at 12 months after enrollment and start of treatment, at this time most patients were off-therapy and a minority of patients with extrapulmonary TB, complicated disease or drug-resistant are at the end of scheduled treatment.

At all time points, samples were collected for metabolomic and immunological analyses. In children exposed to TB, biological samples were only collected at baseline (T0) and at follow-up (T1). Non-TB children were excluded from follow-up (Figure 1).

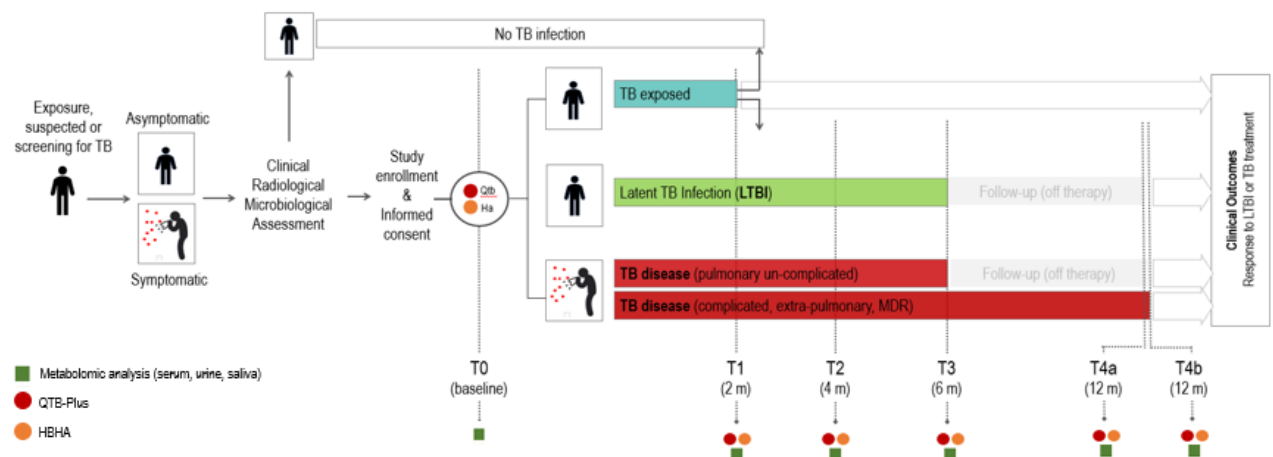


Figure 1. Study design and sample collection timeline for children enrolled in the study.

2.5 Ethical Statement

The study protocol was approved by Ethics Committees of each participating center before starting enrollment (protocol number Naples 50/2023, protocol number Rome 0000905/24). Informed consent was obtained by a trained member of the research team from parents or legal guardians of all candidate participants to the study before any procedure is performed as part of the investigation.

2.6 Immunological analysis

HBHA was purified from *M. smegmatis* pMV3-38 as previously described and batches of purified protein were tested for the presence of LPS using Limulus Amebocyte Lysate QCL-1000TM (Lonza Walkersville, MD USA) (LPS < 1 IU/ml) (30).

The QuantiFERON-TB Gold Plus (QFT-Plus; Qiagen, Venlo, The Netherlands) assay was performed according to the manufacturer's instructions. Briefly, whole blood was collected directly into the QFT-Plus tubes (Nil Control, TB1, TB2, and Mitogen Control) and incubated at 37 °C for 16–24 hours (31). An additional aliquot of whole blood was collected into a separate Nil Control tube, supplemented with 5 µg/mL of recombinant methylated heparin-binding haemagglutinin (HBHA), and incubated under the same conditions (37 °C, 16–24 hours). Following incubation, plasma was harvested by centrifugation and stored at –20 °C until analysis. Interferon-gamma (IFN-γ) concentrations were measured using the QFT ELISA kit (Qiagen, Venlo, The Netherlands). Results are reported in IU/mL after background subtraction using the Nil tube. A positive response was defined as IFN-γ ≥ 0.35 IU/mL for the QFT-Plus assay, in accordance with the manufacturer's guidelines, and ≥ 0.25 IU/mL for the HBHA-based IGRA, based on previously established thresholds in adults (31).

2.7 Untargeted Metabolomic analysis

2.7.1 Metabolite Extraction and Derivatization

Metabolome extraction, purification, and derivatization were conducted using the MetaboPrep GC kit (Theoreo, Montecorvino Pugliano, Italy). Following the manufacturer's protocol, 50 µL of serum sample was added to an Eppendorf microcentrifuge tube containing the extraction mixture and an internal standard. The mixture was vortexed at 1250 rpm for 30 seconds. The extract was then centrifuged at 16000 rpm for 5 minutes at a temperature below 4°C. Two hundred microliters of the supernatant was transferred to another tube containing the purification mixture and vortexed again at 1250 rpm for 30 seconds. A quick centrifugation at 16000 rpm at

temperatures below 4°C allowed for solid sedimentation. One hundred seventy-five microliters of the upper liquid phase were transferred to a glass vial and lyophilized overnight. For derivatization, the lyophilized sample was mixed with 50 µL of the first derivatization reagent and vortexed at 1200 rpm at room temperature for 90 minutes. Then, 25 µL of the second derivatization reagent was added and vortexed at 1200 rpm for another 90 minutes. The derivatized metabolome was transferred to a 100 µL insert for autosampler injection and centrifuged at 16000 rpm for 5 minutes at temperatures below 4°C before GC-MS injection.

2.7.2 GC-MS Analysis

Two microliters of the derivatized solution were injected into a GC-MS system (GC-2010 Plus gas chromatograph coupled to a 2010 Plus single quadrupole mass spectrometer; Shimadzu Corp., Kyoto, Japan). Chromatographic separation was performed using a 30 m CP-Sil 8 CB fused silica capillary column (0.25 mm i.d., 1.00 µm film thickness; Agilent, J&W Scientific, Folsom, CA, USA) with helium as the carrier gas. The oven temperature was initially set to 100 °C and held for 1 minute, then increased at 6 °C/min to 320 °C, followed by a 2.33-minute hold. The gas flow was adjusted to maintain a constant linear velocity of 39 cm/s, and the split ratio was set to 1:5. The mass spectrometer operated in electron impact mode (70 eV) in full-scan mode over a range of 35–600 m/z, with a scan speed of 3333 amu/sec and a solvent delay of 5 minutes. The total GC run time was 40 minutes.

To ensure analytical reproducibility and data quality, GC-MS analyses were performed in batches of 25 samples, each monitored using a comprehensive quality control (QC) protocol. Four types of controls were systematically included in each batch: (i) a solvent blank (2 µL hexane), (ii) a derivatized standard mixture containing 15 known metabolites (including sugars, amino acids, fatty acids, organic acids, and steroids), (iii) a pooled QC sample prepared by combining 2 µL aliquots from 50 randomly selected derivatized study samples, and (iv) a technical replicate of a randomly selected sample within the batch. A batch was considered valid only if all of the following criteria were met: (1) the blank injection showed no detectable peaks, (2) peak areas of all compounds in the standard

mix (normalized to the internal standard) were within 10% of expected values, (3) the coefficient of variation (CV) for the 100 most intense peaks in the technical replicate was below 15%, and (4) the pooled QC sample clustered within 5% of the total variance in a PCA model constructed from all pooled samples.

2.7.3 Metabolite Identification

Metabolite identification followed the methods of Troisi et al. (32), using MS-based spectral deconvolution to accurately separate overlapping signals. Untargeted metabolites were identified by comparing each peak's mass spectrum with the NIST-2014 library collection (NIST, Gaithersburg, MD, USA). The linear index difference tolerance was set to 10, and the minimum matching spectrum library search was set to 85% (level 2 identification according to Metabolomics Standards Initiative [MSI]) (33). Only GC-MS signals detected in at least 80% of the samples were included in the analysis. Metabolite peaks with very low intensity, indicating low concentration and poor mass spectral quality, were not further investigated. Furthermore, signals originating from the same metabolites, such as sugars producing multiple derivatization products, were regarded as separate features. Metabolites identified as relevant were further confirmed at MSI Level=1 using analytical standards.

2.8 Statistical analysis

Data were analyzed using SPSS (SPSS, Chicago, IL) and Prism 5 software (Graphpad Software 5.0, San Diego, CA, USA). Differences in frequencies were evaluated by the Fisher exact test. The median of IFN- γ production was calculated; the non-parametric Mann–Whitney U test was used to compare medians for unpaired comparisons and the Wilcoxon test for paired comparisons; the Kruskal–Wallis test was used to compare medians among the different groups of TB and LTBI patients, including the TB subgroups analyzed.

Normal data distribution was assessed using the Shapiro-Wilk test. Due to non-normal data distribution, the one-way non-parametric heteroscedastic ANOVA (Welch one-way

ANOVA) with Tukey's post hoc test was used for inter-group comparisons of continuous variables. For categorical variables expressed as percentages, statistical significance was assessed using the chi-square (χ^2) test. Differences were considered significant at p values ≤ 0.05 .

The metabolomic statistical analysis of the enrolled subjects' characteristics was performed using R, a language and environment for statistical computing and graphics (34).

GC-MS metabolomics results were summarized in a comma-separated matrix file and analyzed using appropriate statistical software (R - Foundation for Statistical Computing, Vienna, Austria) after chromatographic alignment using the PTW algorithm (35). The chromatographic data for Partial Least Squares Discriminant Analysis (PLS-DA) were organized with one sample per row and one metabolite per column. Normalization procedures involved data transformation and scaling, with data transformation achieved via generalized log transformation and data scaling by autoscaling (mean-centered and divided by the standard deviation of each variable). The chromatographic peak area normalization using the internal standard (2-isopropyl malic acid) was also applied.

Class separations were achieved using PLS-DA, a supervised method employing multivariate regression techniques to extract information via linear combinations of original variables (X) that predict class membership (Y). PLS regression was executed using the `plsr` function in the R `pls` package. Classification and cross-validation were performed using the corresponding wrapper function in the `caret` package. The optimal number of components for each PLS-DA model was determined by cross-validation, selecting the model configuration that maximized the Q^2 value. This approach was used to balance model complexity and generalizability while minimizing overfitting. To enhance the reliability of our data analysis, the Synthetic Minority Over-sampling Technique (SMOTE) algorithm was employed to address class imbalance in categorical variables. In metabolomics, where datasets typically contain many more features than samples, under sampling can lead to substantial information loss. Therefore, SMOTE was used to augment the minority classes without discarding data, helping to stabilize model training while preserving the original dataset structure. This approach allowed us to

construct more balanced and interpretable classification models. A permutation test assessed the significance of class discrimination. For each permutation, a PLS-DA model was built between the data (X) and the permuted class labels (Y) using the optimal number of components determined by cross-validation for the model based on the original class assignment. Two types of test statistics measured class discrimination: prediction accuracy during training and separation distance based on the ratio between “Between” group sum of squares and “Within” group sum of squares (B/W-ratio). If the observed test statistics fell within the distribution based on permuted class assignments, class discrimination was not statistically significant. Variable Importance in Projection (VIP) scores were calculated for each component, representing a weighted sum of squares of the PLS loadings, considering the explained Y-variation in each dimension.

3. Results

3.1 Characteristics of the population

A total of 118 children (53, 44.9% females; median age, 90 months; IQR, 101) were enrolled in the two centers during the study period, with 79 patients recruited at the University of Naples Federico II and 39 at the Gemelli University Hospital in Rome.

Following diagnostic work-up, 4 children (3.4 %) were classified as non-TB, 35 (29.7%) as exposed, while 54 (45.8 %) received a diagnosis of active TB and other 25 (21.2%) were diagnosed with LTBI.

Characteristics of exposed, LTBI and active TB including demographic and clinical data, exposure history, and vaccination status are reported in Table 1. When two-group comparison were conducted, significant differences emerged only for BCG vaccination status between the LTBI and active TB groups ($p = 0.003$) and for recent exposure between the exposed and active TB groups ($p = 0.0005$).

	Exposed n=35	LTBI n=25	active TB n=54	p
Median age, months (IQR)	53 (94)	108 (91)	80.5 (108)	0.003
Female gender (%)	18 (51.4)	9 (36.0)	26 (48.1)	0.470
Italian nationality (%)	12 (34.3)	3 (12.0)	8 (14.8)	0.042
Comorbidity (%)	0 (0)	4 (16.0)	2 (3.7)	0.018
BCG vaccinated (%)	7 (20.0)	9 (36.0)	3 (5.6)	0.022
Recent exposure to the index case (%)	25 (71.4)	14 (56.0)	16 (29.6)	<0.01

Table 1. Characteristics of exposed, LTBI and active TB enrolled participants.

Abbreviations: BCG, Bacille Calmette–Guérin; IQR, interquartile range; LTBI, latent tuberculosis infection; TB, tuberculosis.

Table 2 summarizes sample collection during the study (number of participants providing samples at each time point). A total of 13 exposed, 4 LTBI, and 10 active TB participants

were lost to follow-up. An additional 2 exposed, 8 LTBI, and 15 active TB participants had not yet completed all scheduled visits at the time of analysis. Not all enrolled participants provided samples at every scheduled time point.

	T0	T1	T2	T3	T4
Exposed	35	25	-	-	-
LTBI	24	22	15	16	12
TB	47	42	26	25	20

Table 2. Monitoring of sample collection across study time points.

Values indicate the number of participants who provided samples at each visit; not all enrolled participants contributed samples at every scheduled time point; not all patients completed the follow-up.

Among children with active TB, pulmonary TB accounted for the majority of cases (38, 70.3%), most frequent extrapulmonary presentation was pleural TB (8, 14.8%), followed by central nervous system/miliary TB (5, 9.3%) and tuberculous lymphadenitis (2, 3.7%); one abdominal TB case was observed (1, 1.8%).

Overall, pulmonary involvement was present in most patients (51 [94.4%]), either as pulmonary TB alone or concomitant with extrapulmonary disease. Among children with pulmonary involvement, 7 (13.7%) required CT to reach a definitive diagnosis.

Slightly more than half (29, 53.7%) presented with typical TB-related symptoms, and 22 (40.7%) had severe disease.

Twenty-six cases (48.1%) were microbiologically confirmed—2 by Xpert MTB/RIF Ultra alone, 6 by culture alone, and 18 by both—with 2 of the both-positive patients also Ziehl–Neelsen smear-positive. Three cases were isoniazid-resistant (5.6%); none were rifampicin-resistant and no MDR-TB was detected.

Among 54 patients, 22 (40.7%) were cured with standard therapy, 8 (14.8%) after prolonged treatment, 3 (5.6%) with a regimen modified for resistance detected after treatment initiation, and 7 (13.0%) with therapy modified due to adverse reactions.

Ten (18.5%) were lost to follow-up, 4 (7.4%) had ongoing therapy at last follow-up; no deaths occurred. The clinical characteristics, microbiological findings, and treatment outcomes of children with active tuberculosis are summarized in Table 3.

	n (%)
Localization	
Pulmonary TB	38 (70.3)
Extrapulmonary TB	16 (29.7)
• Pleural TB	8 (14.8)
• CNS / Miliary TB	5 (9.3)
• Tuberculous lymphadenitis	2 (3.7)
• Abdominal TB	1 (1.8)
Disease presentation	
CT required for diagnosis	7 (13.7)
TB-related symptoms	29 (53.7)
Severe disease	22 (40.7)
Microbiological confirmation	26 (48.1)
• Xpert MTB/RIF Ultra only	2 (3.7)
• Culture	6 (11.1)
• Both Xpert and culture	18 (33.3)
Drug resistance	
• Isoniazid-resistant	3 (5.6)
• Rifampicin-resistant	0 (0)
• MDR	0 (0)
Treatment outcomes	
• Cured with standard therapy	22 (40.7)
• Cured after prolonged treatment	8 (14.8)

• Modified regimen for resistance	3 (5.6)
• Modified regimen due to adverse reactions	7 (13.0)
• Lost to follow-up	10 (18.5)
• Ongoing therapy at last follow-up	4 (7.4)
• Deaths	0 (0)

Table 3. Clinical characteristics and outcomes of children with active tuberculosis (n=54).

Abbreviations: TB, tuberculosis; CNS, central nervous system; CT, computed tomography; MDR-TB, multidrug-resistant tuberculosis.

3.2 Immunological analysis

3.2.1 QFT and HBHA-based IGRA response in the study population

We conducted preliminary immunological analyses at T0 in 27 exposed contacts, 19 children with LTBI, and 43 with active TB.

QFT did not discriminate LTBI from active TB based on IFN- γ medians (TB1 p:0.831; TB2 p:0.577). In contrast, the HBHA-based IGRA showed higher IFN- γ responses in LTBI (0.69 [2.15] IU/mL) than in active TB (0.18 [1.03] IU/mL)—though these differences narrowly missed statistical significance (p:0.050). Notably, the LTBI median lay above the assay cut-off (0.25 IU/mL), whereas the active TB median remained below it (Figure 2). Moreover, a greater proportion of LTBI cases tested positive than active TB cases (14/19 [73.7%] vs 19/43 [44.2%]; p = 0.052), also narrowly missing the significance threshold.

Among exposed contacts, 15/27 (55.6%) were HBHA-IGRA-positive; their median IFN- γ response (0.49 [1.16] IU/mL) exceeded the cut-off, with no significant differences versus LTBI (p = 0.210) (Figure 2).

Overall, the HBHA-based IGRA showed a consistent trend toward higher reactivity in LTBI.

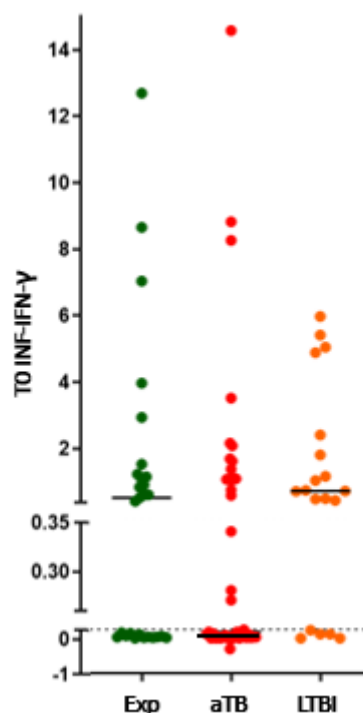


Figure 2. IFN- γ response to HBHA in exposed, LTBI and active TB children.

Each point on the graph represents an individual result. Exposed individuals are represented by green dots, LTBI by orange dots, and active TB by red dots. Horizontal lines indicate the median of IFN- γ production. Dotted line indicates the cut-off for HBHA (0.25 UI/ml). p-values are reported in the main text. *Abbreviations:* Exp exposed children; LTBI, latent TB infection; active TB, active TB; HBHA, heparin-binding haemagglutinin.

3.2.2 HBHA responsiveness in asymptomatic and milder active TB

Within the active TB cohort, HBHA responses were detected more often in asymptomatic than symptomatic children—12/22 (54.5%) vs 7/21 (33.3%; $p = 0.161$)—and median HBHA-induced IFN- γ tended to be higher in asymptomatic cases (0.53 [1.37] vs 0.12 [0.40] IU/mL; $p = 0.117$) (Figure 3, Panel A). A similar pattern was observed by disease severity: positivity was more frequent in non-severe than severe cases—14/26 (53.8%) vs 5/17 (29.4%; $p = 0.115$)—with higher IFN- γ (0.31 [1.33] vs 0.11 [0.39] IU/mL; $p = 0.296$) (Figure 3, Panel B).

Importantly, when compared to the assay cut-off, the median HBHA-induced IFN- γ was above the cut-off in asymptomatic and non-severe cases but remained below the cut-off in symptomatic and severe cases (Figure 3, Panel B).

Finally, in the microbiological stratification, HBHA positivity was observed in 7/19 confirmed cases and 12/24 unconfirmed cases (36.8% vs 50.0%; $p = 0.388$), with no significant difference, while the median HBHA-induced IFN- γ was below the cut-off in both microbiologically confirmed and unconfirmed subgroups (0.11 [1.05] vs 0.23 [1.01] IU/mL; $p = 0.419$) (Figure 3, Panel C).

Taken together, these analyses indicate a trend toward preserved HBHA responsiveness in asymptomatic and non-severe active TB, although the differences did not reach statistical significance, with no evidence of association with bacillary load.

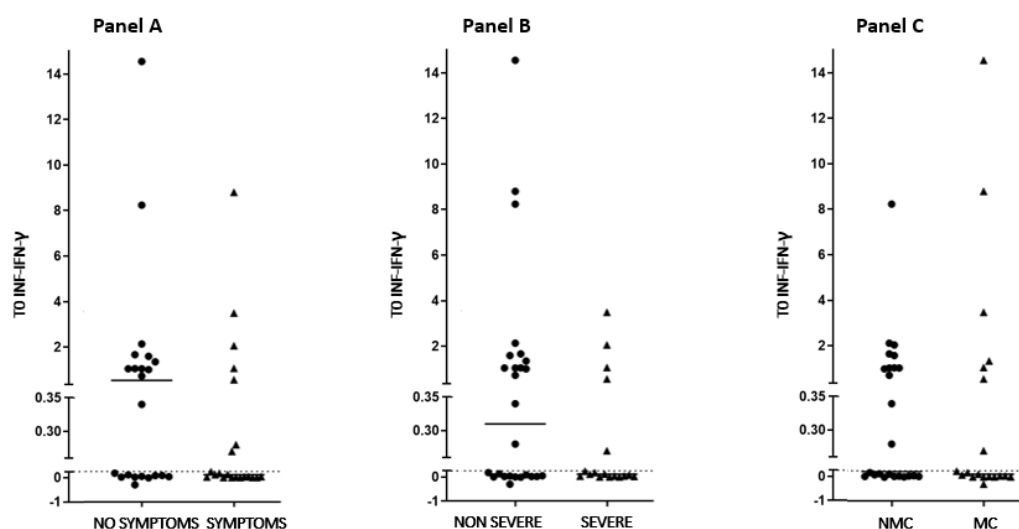


Figure 3. IFN- γ response to HBHA among active TB subgroups.

Each point on the graph represents an individual result. Horizontal lines indicate the median of IFN- γ production. Dotted line indicates the cut-off for HBHA (0.25 UI/ml). p -values are reported in the main text. Panel A: children with asymptomatic active TB are represented by dots, children with symptomatic active TB by triangles.

Panel B: children with non-severe active TB are represented by dots, children with severe active TB by triangles.

Panel C: children with non-microbiologically confirmed active TB are represented by dots, children with microbiologically confirmed active TB by triangles.

Abbreviations: active TB, active TB; HBHA, heparin-binding haemagglutinin, NMC: non microbiologically confirmed, MC: microbiologically confirmed

3.2.3 Longitudinal Responses of HBHA During Treatment for active TB

Plasma IFN- γ responses to HBHA were measured at scheduled time points among active TB. HBHA-induced IFN- γ already increased from T0 (0.18 [1.03] IU/mL) to T1 (0.43 [1.43] IU/mL), converting from negative to positive (p:0.229) (Figure 4).

HBHA-induced IFN- γ was preserved over the course of treatment (T2: 0.69 [2.76] IU/ml; T3: 0.275 [1.75] IU/ml) and at the end of follow-up (T4:0.425 [0.15] IU/ml) though the median values fluctuated, with a non-statistically significant difference in medians between one time point and the next (Figure 4).

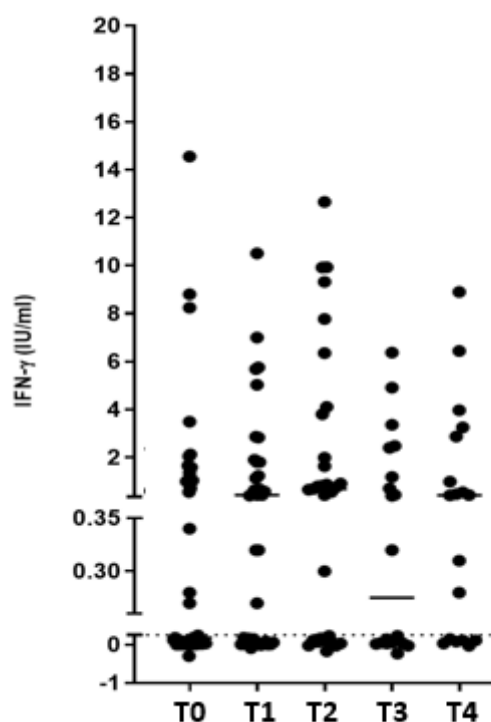


Figure 4. Longitudinal IFN- γ response to HBHA during treatment in active TB.

Each point on the graph represents an individual result. Horizontal lines indicate the median of IFN- γ production. Dotted line indicates the cut-off for HBHA (0.25 UI/ml). p-values are reported in the main text. On the x-axis, the different sampling time-points are shown. T0: at diagnosis; T1: 2 months after diagnosis; T2: 4 months after diagnosis; T3: 6 months after diagnosis; T4: 12 months after diagnosis. *Abbreviations:* active TB, active TB; HBHA, heparin-binding haemagglutinin.

INF- γ positive response to HBHA stimulation improved over time, increasing already between T0 and T1 (from 19/43 [44.2%] at T0 to 25/39 [64.1%] at T1) and remained relatively stable thereafter (Table 4).

T0	T1	<i>p</i>	T2	<i>p</i>	T3	<i>p</i>	T4	<i>p</i>
19/43 [44.2%]	25/39 [64.1%]	0.081	20/31 [64.5%]	1.0	10/20 [50.0%]	0.386	12/18 [66.7%]	0.341

Table 4. Proportion of HBHA-IGRA–positive patients over time (T0–T4).

4. Metabolomic analysis

We hereby present the preliminary metabolomic analyses performed on 49 serum samples (25/35 exposed, 10/25 LTBI, 14/54 active TB) collected at diagnosis (T0). The study will be extended to include all enrolled patients, longitudinal sampling at subsequent time points, and analyses of additional biospecimens collected (urine and saliva).

The PLS-DA model revealed distinct clustering of exposed, LTBI, and active TB children, suggesting that each group is characterized by a specific metabolic signature.

The active TB group was clearly separated from the others, indicating substantial metabolic perturbations associated with active disease. Children with LTBI displayed an intermediate metabolic pattern, partially overlapping with the exposed group yet trending toward the active TB cluster, consistent with a continuum of metabolic

alterations from exposure to latent infection and active disease (Figure 5).

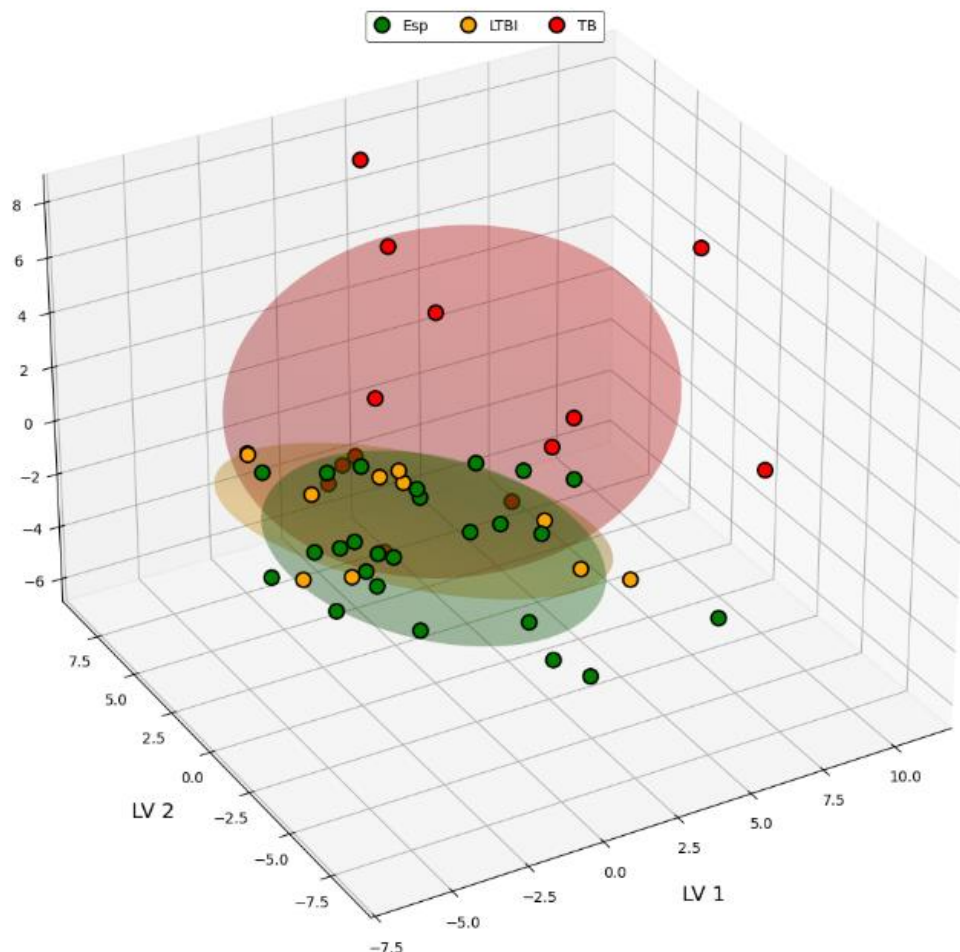


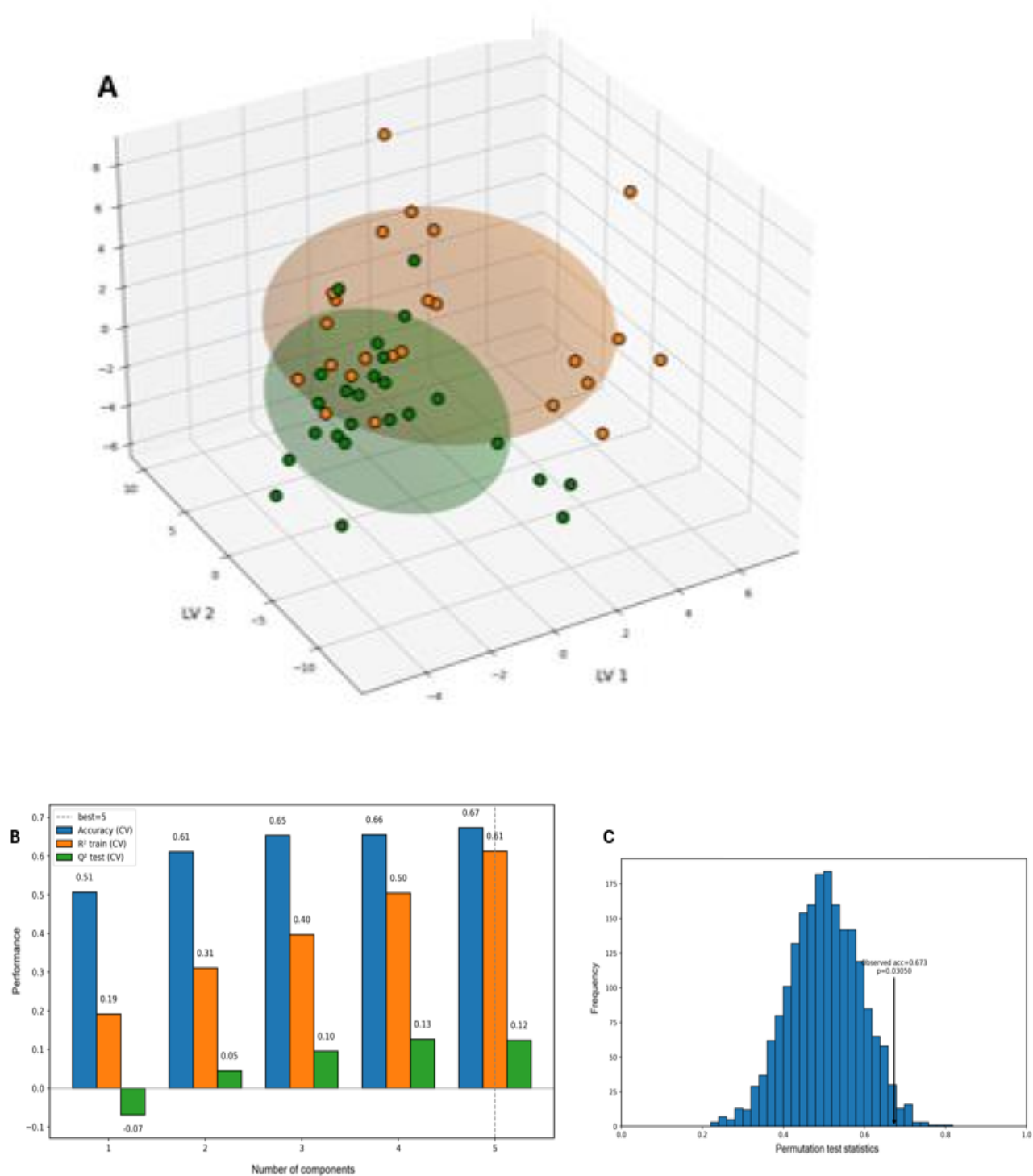
Figure 5. Serum metabolomic signature in exposed, LTBI, and active TB children at diagnosis (T0). Partial Least Square-Discriminant Analysis (PLS-DA) classification model discerning exposed children (green dots), from LTBI (orange dots) and active TB (red dots) at diagnosis.

Subsequently, children with tuberculosis infection (LTBI and active TB) were compared to exposed but uninfected children. The PLS-DA model confirmed a clear discrimination between the two groups, underscoring the robustness and reproducibility of the observed metabolic differences. Within the TB cluster, two distinct subclusters were observed, suggesting the existence of two different metabolomic phenotypes among infected children (Figure 6, Panel A).

The best diagnostic performance of the PLS-DA classification model was achieved using a five-component model, selected as optimal based on cross-validation. The permutation test statistically validated the class separation between exposed children and those with tuberculosis infection. Performance indices based on the five-components model were: $R^2Y = 0.61$, $Q^2Y = 0.12$, and the significant $p = 0.03$ calculated on 2000 permutations (Figure 6, Panel B and C).

Several metabolites showed a VIP score greater than 1.6, identifying them as key discriminant variables contributing to the observed group separation in the PLS-DA model. Among these, isoleucine, glyceric acid, glycine, and serine exhibited a p -value < 0.05 (t-test) and were all average less concentrated in children with tuberculosis infection compared with exposed controls (Figure 6 Panel D).

Metabolite set enrichment analysis performed including all differently expressed metabolites (Figure 6, Panel E), allowed to identify glyoxylate and dicarboxylate, glycine, serine and threonine metabolism, ascorbate and aldarate metabolism and galactose metabolism as the top enriched pathways (Figure 6, Panel E).



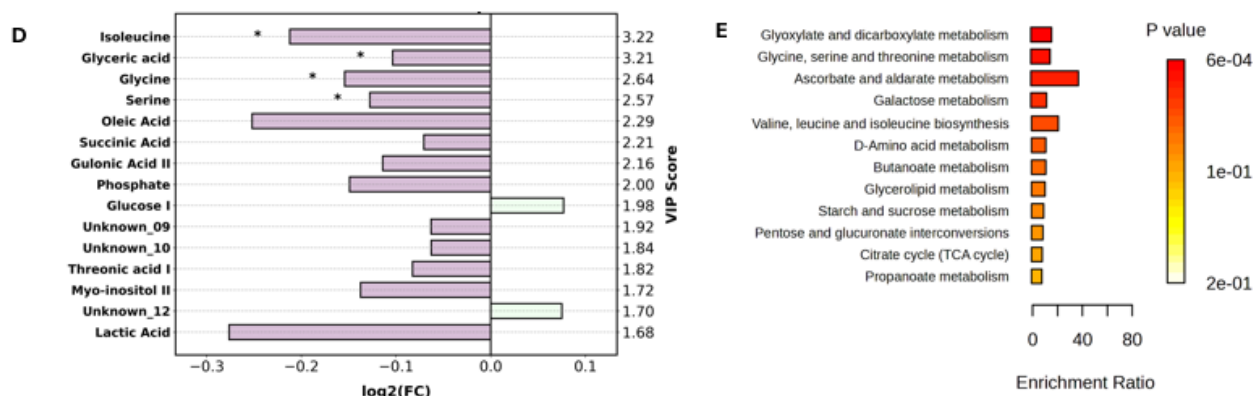


Figure 6. Serum metabolomic signature in exposed and tuberculosis-infected (LTBI and active TB) children at diagnosis (T0).

Panel A. Partial Least Square-Discriminant Analysis (PLS-DA) classification model discerning exposed children (green dots), from tuberculosis-infected -LTBI and active TB- (orange dots) at diagnosis. (B) Diagnostic performance of the PLS-DA classification model when using different numbers of latent variable (number of components, x axis). The best performance (denoted with a dashed vertical line) is achieved when using five variables as the basis for classification. (C) PLS-DA model validation by permutation test based on separation distance. The p-value based on 2000 permutations is $p=0.03$. (D) The first 15 metabolites ranked according to their Variable Importance in Projection (VIP) scores are reported. The increase or decrease in serum levels of each metabolite in exposed and tuberculosis-infected children is expressed as the logarithmic fold change ($\log_2FC$). Purple horizontal bars to the left indicate higher levels in exposed children, whereas blue horizontal bars to the right indicate higher levels in tuberculosis-infected children. Asterisks denote metabolites showing statistical significance ($p < 0.05$) in the t-test. (E) Metabolite Set Enrichment Overview performed using the metabolites (panel d) identified. The length of each bar express the degree of enrichment ratio and the color scale reflects the p-values.

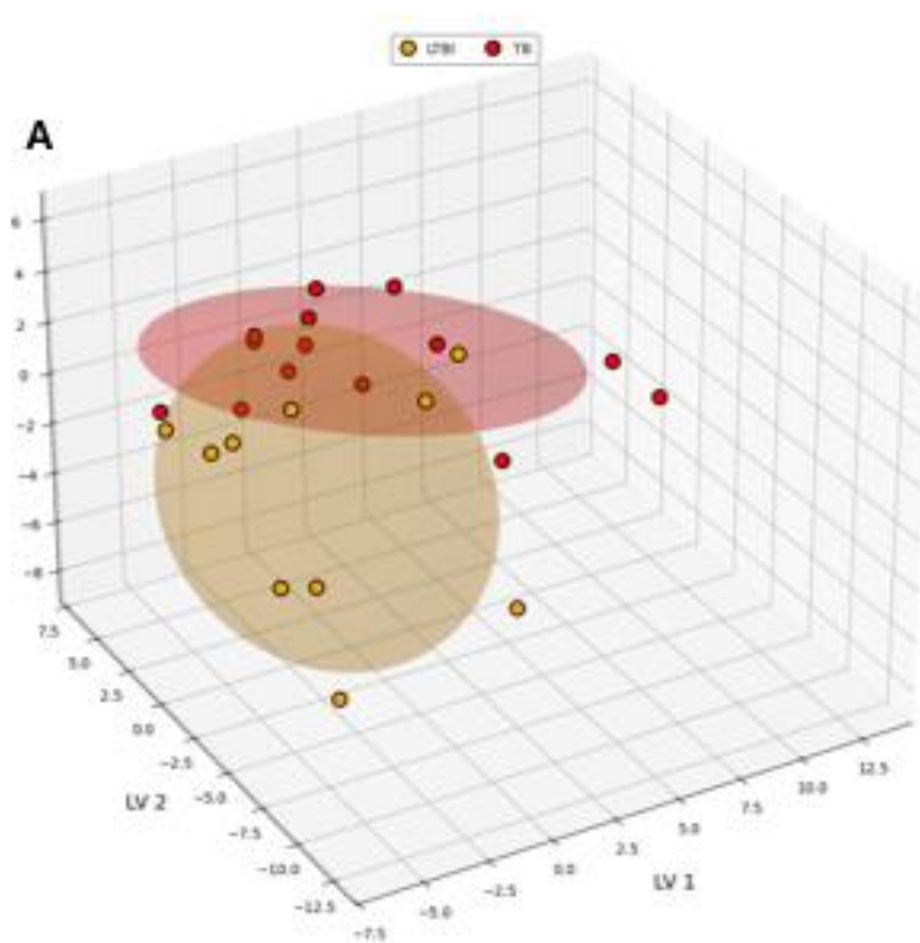
Finally, children with LTBI were compared to active TB. The PLS-DA model showed a clear class separation between the two groups (Figure 7, Panel A).

The best diagnostic performance of the PLS-DA classification model was achieved using a two-component model, selected as optimal based on cross-validation. The permutation test statistically validated the class separation between LTBI and those with active TB. Performance indices based on the five-components model were: $R^2Y = 0.51$, $Q^2Y = 0.34$, and $p = 0.08$ (Figure 7, Panel B and C).

Several metabolites showed a VIP score greater than 1.5, identifying them as key discriminant variables contributing to the observed group separation in the PLS-DA model. Among these, 2-hydroxyglutaric acid, succinic acid and threonic acid III exhibited

a p -value < 0.05 (t-test) and were all average increased in children with active TB compared with LTBI (Figure 7 Panel D).

Metabolite set enrichment analysis performed including all differently expressed metabolites (Figure 7, Panel E), allowed to identify pentose and glucuronate interconversions, pentose phosphate pathway and galactose metabolism as the top enriched pathways (Figure 7, Panel E).



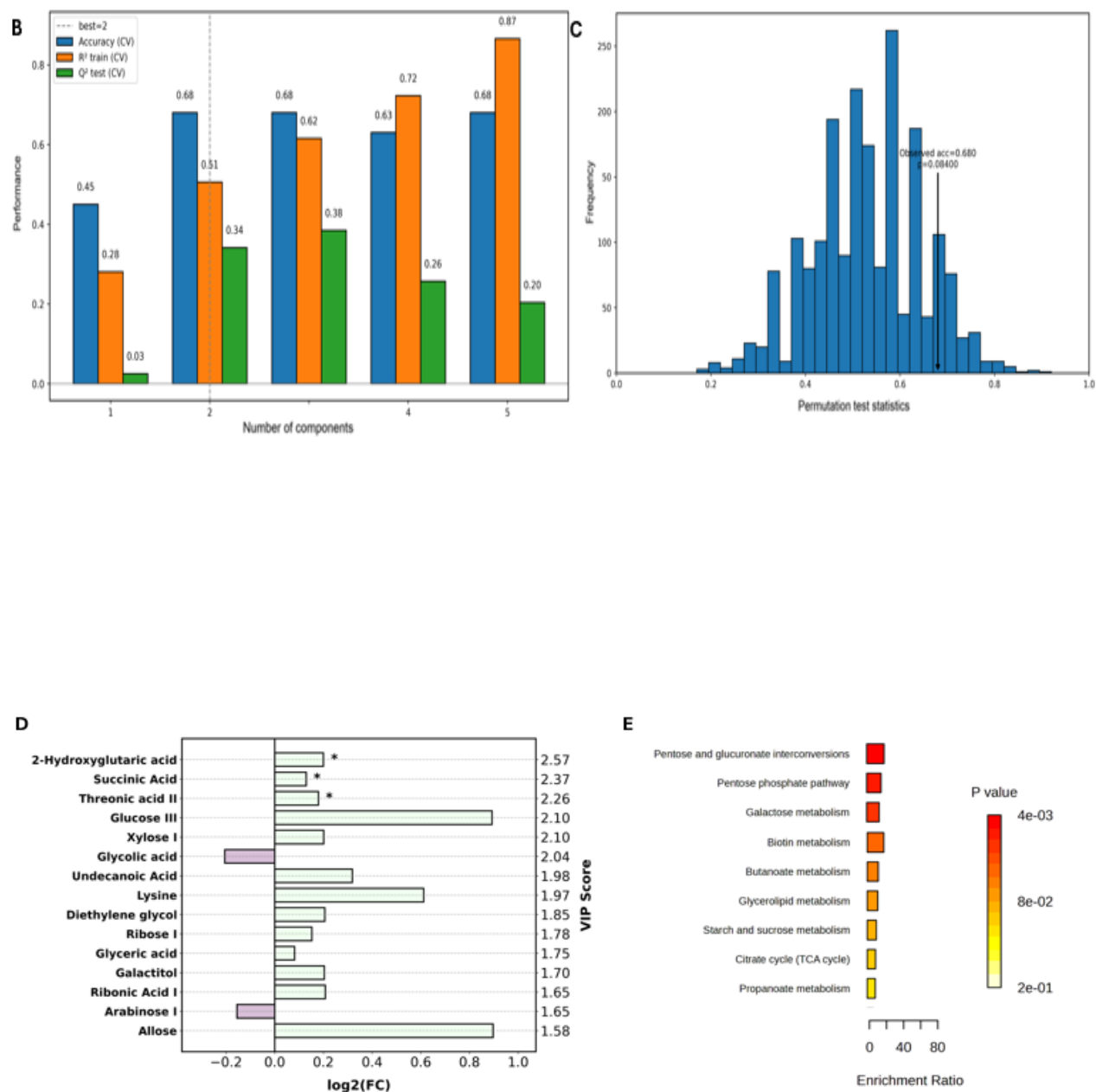


Figure 7. Serum metabolomic signature in LTBI and active TB children at diagnosis (T0).

Panel A. Partial Least Square-Discriminant Analysis (PLS-DA) classification model discerning LTBI children (orange dots), from active TB (red dots) at diagnosis. (B) Diagnostic performance of the PLS-DA classification model when using different numbers of latent variable (number of components, x axis). The best performance (denoted with a dashed vertical line) is achieved when using two variables as the basis for classification. (C) PLS-DA model validation by permutation test based on separation distance. The p-

value based on 2000 permutations is $p=0,08$. (D) The first 15 metabolites ranked according to their Variable Importance in Projection (VIP) scores are reported. The increase or decrease in serum levels of each metabolite in LTBI and active TB children is expressed as the logarithmic fold change ($\log_2FC$). Purple horizontal bars to the left indicate higher levels in LTBI children, whereas blue horizontal bars to the right indicate higher levels in active TB children. Asterisks denote metabolites showing statistical significance ($p < 0.05$) in the t-test. (E) Metabolite Set Enrichment Overview performed using the metabolites (panel d) identified. The length of each bar expresses the degree of enrichment ratio and the color scale reflects the p-values.

4. Discussion

To our knowledge, this is the first study to integrate metabolomic and immunological analyses in a cohort of pediatric patients with tuberculosis.

A combined immunological and metabolomic analysis provides a more comprehensive understanding of pediatric TB. Immunological assays reveal the host T-cell response to *Mycobacterium tuberculosis* antigens, while metabolomic profiling captures the systemic metabolic perturbations caused by infection. The rationale for combining these modalities is that immunological markers and metabolite signatures may complement each other, potentially improving the differentiation between latent and active tuberculosis as well as among the stages across the disease spectrum.

In our cohort, children with LTBI showed tendentially stronger IFN- γ responses to HBHA stimulation than children with active TB disease. Both the median IFN- γ concentration and the proportion of HBHA-IGRA-positive responders were higher in LTBI than in active TB, notably the LTBI median was above the assay cut-off (0.25 IU/mL) and the active TB median below it; however, these differences narrowly missed statistical significance. This trend aligns with the hypothesis that LTBI subjects mount a stronger memory T-cell response to latency-associated antigens such as HBHA, whereas active TB is associated with a loss or reduction of HBHA reactivity (36). HBHA has been investigated more extensively in adults than in children. However, pediatric populations are precisely where enhanced diagnostic performance could be most beneficial, due to the challenge of differentiating active from LTBI on clinical, radiologic, and microbiologic criteria (9,10,37).

In a pediatric Chinese cohort Wen *et al.* demonstrated that IGRA using methylated HBHA were lower in active TB patients than in LTBI individuals, regardless of disease site or microbiological confirmation, even in BCG-vaccinated population (38).

The trend we identified is also consistent with Sali *et al.* who reported that 91.1% of QFT-positive children with LTBI had a positive HBHA-based IGRA, compared to only 31.6% of children with active TB, in Italy ($p = 0.001$). LTBI cases also showed a significantly higher median IFN- γ response than active TB cases. Notably, in that study, the few active TB children showing HBHA-induced IFN- γ were mostly asymptomatic

cases (18). More recent pediatric work by Buonsenso *et al.* (2023) further confirmed this observation (39). We observed a similar pattern; within our active TB group, there was a trend toward higher HBHA-induced IFN- γ responses in asymptomatic and non-severe cases, with median values above the 0.25 IU/mL cut-off, similarly to what observed in children with LTBI. Whereas symptomatic or severe TB cases showed medians below this threshold. This pattern suggests an inverse relationship between disease severity or symptomatology and HBHA-specific IFN- γ responses. Children with milder disease appear to retain HBHA-specific immunity, while those with severe TB show reduced responses. Therefore, HBHA positivity could serve as an immunological hallmark of a contained infection.

We may speculate on the potential role of measuring IFN- γ responses to HBHA antigens in children with TB infection as a means to better differentiate those with active TB from those with LTBI.

Our initial aim was to assess whether this specific IFN- γ response could help identify children with incipient TB infection—namely, those who meet the current immunological criteria for TB infection but show no radiological or clinical evidence of active TB and may be at risk of rapid disease progression, or those with mild pulmonary or lymph node involvement in the absence of overt symptoms or signs of active TB. However, our data suggest that the HBHA response does not currently fulfill this discriminative role, as a comparable proportion of asymptomatic active TB and LTBI patients showed values above the established cut-off.

Another potential clinical application of this assay might be to support the diagnosis of active TB in cases where conventional microbiological investigations fail to isolate *Mycobacterium tuberculosis*, thus precluding a confirmed diagnosis. This scenario is particularly relevant in pediatric cohorts, where microbiological confirmation is achieved in only a minority of cases. In our study, IFN- γ responses to HBHA were similarly reduced in both microbiologically confirmed and unconfirmed TB cases, with median values falling below the defined cut-off.

In our longitudinal sub-analysis of active TB cases, HBHA-induced IFN- γ levels increased from diagnosis to the first follow-up at 2 months after treatment initiation, coinciding with the transition from the intensive to the continuation phase of therapy. The proportion of HBHA-positive patients also rose from 44.2% to 64.1%. This indicating a trend toward restoration of HBHA-specific T-cell function with improving control of infection (though this change did not reach statistical significance).

This tendency parallels previous observations: Sali et al. reported that most children with active TB who initially exhibited a poor HBHA-induced IFN- γ response developed a detectable IFN- γ response to HBHA by the end of therapy (18), and Buonsenso et al. reported a significant increase in median HBHA responses during treatment, with the proportion of positive HBHA-based IGRA results rising from 19/43 at diagnosis to 25/28 at completion (39).

Although our findings did not reach statistical significance, they are consistent with the hypothesis that HBHA-based IGRAs could have value in therapy monitoring and could serve not only as a diagnostic adjunct but also as a treatment-monitoring tool in children, signaling immune recovery during treatment and inform treatment completion decisions (19,39).

It should be noted that, unlike many other bacterial infections, where monitoring C-reactive protein (CRP) or procalcitonin can support discontinuation of antibiotics decisions, tuberculosis lacks validated inflammatory markers to guide treatment cessation. This underscores the need for reliable biomarkers of treatment response, particularly in children, in whom radiation-protection considerations limit repeated imaging and most cases are microbiologically unconfirmed, making documentation of culture conversion infeasible. Notably, at a time when the most recent international guidelines advocate shortened treatment durations for non-severe pediatric TB (28), the use of HBHA-based IGRAs and could have particularly valuable applications, both in helping to define non-severe disease at diagnosis and in guiding therapeutic decisions, including duration adjustment.

The detection of HBHA-induced IFN- γ responses in asymptomatic active TB limits the clinical utility of HBHA-based assays, because the key pediatric challenge is to distinguish LTBI from asymptomatic active disease. Although combining QFT-Plus

with HBHA-IGRA may provide a more nuanced immunologic profile, meaningful clinical translation will likely require additional, complementary biomarkers to reliably separate latent infection from early disease. In this context, metabolomics offers a promising source of host-derived signals that could refine such discrimination while also enhancing treatment monitoring. Integrating immunological markers with metabolite panels might improve performance, since the two types of biomarkers reflect different aspects of the host-pathogen interaction.

Our serum untargeted metabolomic analysis, derived from on a subset of samples collected at diagnosis, revealed - in a three-group comparison (exposed contacts, LTBI, and active TB) - a clear separation of children with active TB from the other groups. Children with LTBI exhibited an intermediate profile that partially overlapped with exposed contacts yet trended toward the active TB cluster, consistent with a progressive host metabolic adaptation from exposure through latency to overt disease, across the TB spectrum. Compared with exposed but uninfected contacts, children with *M. tuberculosis* infection (LTBI and active TB) show a significantly different serum metabolic profile ($p:0.03$). Several metabolites contributed strongly to group discrimination ($VIP > 1.6$) and were individually significant ($p < 0.05$), including isoleucine, glyceric acid, glycine, and serine, which were on average lower in infected children than in exposed contacts. Additional metabolites reduced in the infected group included oleic acid, succinic acid, gluconic acid, phosphate, threonic acid, myo-inositol, and lactic acid. Pathway enrichment analysis implicated glyoxylate and dicarboxylate metabolism, glycine/serine/threonine metabolism, ascorbate and aldarate metabolism, and galactose metabolism. These alterations could indicate a depletion of aminoacids and carbohydrate-related intermediates in infected children and may reflect increased metabolic demands during infection.

The metabolomics literature in tuberculosis remains relatively scarce, and not all studies explicitly compare exposed but uninfected individuals with those who are infected (LTBI and active TB). Even when specific biomarkers differ from ours, several reports converge on disruptions in protein and amino-acid metabolism. In a cohort of Indian children was reported increased kynurenine-to-tryptophan ratios in a targeted analysis with active TB compared exposed children, achieving ~81.5% sensitivity for active TB

diagnosis in their study. Elevated kynurenine and reduced tryptophan suggested activation of the indoleamine-2,3-dioxygenase (IDO-1) pathway as part of the immune-metabolic response (22).

Using a targeted panel in pediatric cohorts, enrolling children with active TB, LTBI, non-mycobacterial pneumonia, and healthy controls, Druszczynska et al. found leucine and kynurenine to be elevated, with citrulline and glutamine reduced, in both active TB and LTBI compared with controls (23).

In children, Sharma et al. reported decreased histidine, glycerophosphocholine, creatine/phosphocreatine, acetate, and choline in targeted profiling of active TB versus healthy controls, and identified seven discriminatory metabolites in untargeted analyses (N- α -acetyl-lysine, polyunsaturated fatty acids, phenylalanine, lysine, lipids, glutamate + glutamine, and dimethylglycine). The authors suggest that these perturbations collectively point to impaired protein synthesis, compromised anti-inflammatory and cytoprotective pathways, abnormalities in energy generation and membrane metabolism, and dysregulated fatty-acid and lipid metabolism in pediatric TB (40).

It should be taken into account that, with the exception of Sharma et al., which coupled an untargeted discovery phase with targeted validation, the pediatric studies summarized above relied predominantly on targeted metabolomics. In adults, an untargeted study comparing asymptomatic exposed but uninfected contacts with active TB (Albors-Vaquer et al.) showed that active TB was characterized by lower levels of amino acids (alanine, lysine, glutamate, glutamine) and intermediates such as citrate and choline (41).

When comparing children with LTBI and those with active TB the PLS-DA model indicated a trend toward separation not statistically significant. Nevertheless, several metabolites showed individual significance ($p < 0.05$), including higher concentrations of 2-hydroxyglutaric acid, succinic acid, and threonic acid in active TB, while glycolic acid and arabinose were relatively increased in LTBI. Pathway enrichment analysis highlighted pentose and glucuronate interconversions, the pentose phosphate pathway, and galactose metabolism, all of which are related to carbohydrate turnover and redox homeostasis. These pathways are related to maintain energy balance and support biosynthetic needs during immune activation. The higher levels of certain organic acids

in active TB may reflect an intensified metabolic demand and mitochondrial stress linked to active infection.

To our knowledge, no previous pediatric metabolomic studies have specifically compared children with LTBI to those with active TB. Among available reports, the study by Druszczyńska et al. represents the only work that explicitly included both LTBI and active TB pediatric groups. Notably, both LTBI and active TB groups showed similar metabolic alterations relative to controls, including elevated leucine and kynurenine and reduced citrulline and glutamine concentrations, while LTBI was additionally characterized by a decrease in valine in stimulated cultures. However, due to the small number of active TB cases ($n = 15$), the authors could not identify a metabolite signature capable of clearly discriminating LTBI from active TB, concluding that metabolic changes were largely shared across the infection spectrum (23).

In adults, Cho et al. used targeted serum metabolomics to compare patients with active TB with individuals with LTBI and healthy controls. They identified a principal discriminant signature characterized by decreased glutamine and increased glutamate in active TB versus LTBI, reflecting a reciprocal shift in amino acid metabolism consistent with TB-associated protein catabolism (42).

From China, an untargeted study profiled adults with LTBI, active TB, and healthy controls. The authors identified 65 metabolites differing across the four groups. Inosine and a specific prostaglandin E1 metabolite were elevated in LTBI, suggesting potential diagnostic markers for latent infection. Cotinine showed a stepwise increase from healthy to LTBI to active TB, indicating possible utility as a progression-risk biomarker from latent to active disease (43).

Taken together, our results suggest that pediatric TB progression involves measurable alterations in serum metabolites. These findings, albeit preliminary, provide a proof of principle for the usefulness of metabolite profiling as an adjunct in the diagnosis of TB in children. We expect that incorporating the additional samples already collected at baseline and the longitudinal follow-up specimens into the analysis will improve discriminatory performance and yield more robust insights.

Strength and Limitations

This work is, to our knowledge, the only pediatric investigation that explicitly integrates immunological and metabolomic assessments within the same cohort, thereby offering a multidimensional view of the host response to *Mycobacterium tuberculosis*.

Regarding the metabolomic component, we adopted an untargeted metabolomic approach to enable an unbiased survey of metabolic alterations in pediatric TB, a field with limited prior data. This discovery-oriented design maximizes coverage and facilitates the identification of novel pathways and candidate biomarkers that targeted panels might miss. The use of an untargeted strategy represents a strength of our work, as relatively few studies have applied untargeted metabolomics in pediatric TB, thereby adding unique exploratory value. Accordingly, our findings should be viewed as hypothesis-generating, to be validated with targeted assays.

The three-group design, comprising exposed but uninfected contacts, children with LTBI, and those with active disease, allows exploration of metabolic variation across the full TB spectrum, rather than relying solely on binary comparisons. The use of samples collected at diagnosis (T0) minimizes potential confounding due to treatment or disease resolution, capturing early infection-associated metabolic signatures. In parallel, systematic collection of follow-up samples can augment these baseline findings and providing insight into disease progression and treatment response.

Nevertheless, several limitations must be acknowledged. The overall sample size was modest, probably explaining why several observed differences remained trends rather than significant results, and further stratification by clinical phenotype, further reduced statistical power. A related issue is that not all children contributed samples at every timepoint, and not all completed the full follow-up schedule, thereby interfering with longitudinal assessments. In addition, a minority of active TB cases were microbiologically confirmed, which raises the possibility of diagnostic misclassification (a few children categorized as “active TB” might actually have been LTBI). We tried to minimize this by rigorous diagnosis and follow-up, but some uncertainty remains. Such misclassification would bias against finding differences, tending to attenuate between-group differences.

Finally, the absence of a comparator group with other respiratory infections limits interpretation of the metabolomic signatures, as some alterations may reflect non-specific response to infection rather than tuberculosis-specific pathways.

Conclusions and future directions

Future work will focus on expanding the present findings. The immediate next step involves extending the metabolomic and immunologic analyses to the full cohort, both at baseline and longitudinal sampling, to strengthen statistical power and assess dynamic changes over time.

In a specific subset of children—particularly those classified as incipient, subclinical and LTBI—we plan to perform additional immunological investigations to better characterize the host response to *M. tuberculosis*. Specifically, supernatants collected after stimulation ex vivo of whole blood with RD1 antigens (e.g., ESAT-6/CFP-10) and HBHA; will be quantified for cytokines other than IFN- γ .

In parallel, peripheral blood mononuclear cells (PBMCs) will undergo antigen stimulation followed by flow cytometry to enumerate *M. tuberculosis*-specific IFN- γ -producing CD4⁺ and CD8⁺ T cells (with unstimulated and mitogen controls). These studies aim to refine the immunological profile associated with infection stages across TB spectrum and to identify early markers of progression or containment.

Regarding the metabolomic, ongoing work will include complementary analyses of urine and saliva to evaluate cross-matrix consistency of the identified metabolic signatures, by exploring whether the pathways identified in serum have correlates in urine or saliva. Importantly, non-invasive biomarkers derived from these samples would have substantial clinical utility, with particular relevance for pediatric populations.

Moreover, analysis of longitudinal samples already collected from these children at multiple time points, including serial serum, urine, and saliva specimens, could determine whether the observed metabolic perturbations normalize with successful therapy, thereby corroborating our baseline insights and possibly identifying biomarkers of treatment response. Finally, the next phase will also introduce a comparator group with other respiratory infections, which will help distinguish TB-specific metabolic responses from general signatures of inflammation or infection.

The final objective will be the integration of immunologic and metabolomic parameters into a combined biomarker algorithm, which could yield a more accurate classifier for pediatric TB than any single measure alone, with HBHA-IGRA providing information beyond conventional IGRA assays, and metabolites add complementary discriminatory value. Ultimately, the aim is to support differential diagnosis between active TB and LTBI, predict incipient TB to enable early identification of children at highest risk of progression, and refine the classification of less severe active TB. This integrative approach could also inform personalized therapy and follow-up. Monitoring immuno-metabolic markers during therapy may help identify patients achieving immune and metabolic normalization, such as recovery of HBHA responsiveness or normalization of specific metabolites, who might be suitable candidates for shortened regimens. Conversely, patients showing persistently altered immune or metabolic profiles could benefit from extended or intensified treatment. Ideally, non-invasive biomarkers derived from urine or saliva would be of particular clinical value, especially in pediatric populations, by facilitating longitudinal monitoring and improving patient adherence. In conclusion, we believe that an integrated immunological and metabolomic analysis is a powerful approach to pediatric TB research. While further expansion and more robust data are required, our work represents a step toward moving beyond current diagnostic paradigms to develop multidimensional biomarker-based tools for early detection, risk stratification, and individualized therapy.

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CHAPTER 6

CONCLUSIVE REMARKS

This work offers an integrated view of pediatric TB, encompassing its epidemiological landscape in a low-incidence country, diagnostic challenges, and the potential of innovative immunological and metabolomic approaches to refine disease diagnosis and management.

The epidemiological analyses from our pediatric regional reference center in Southern Italy revealed how pediatric TB continues to represent a relevant public health concern even in low-incidence settings, while remaining a leading cause of morbidity and mortality in high-incidence countries. The increasing proportion of cases among children of migrant background and the heightened disease severity observed during the COVID-19 pandemic highlighted the fragile balance between surveillance, accessibility of care, and social factors. These findings underscore the importance of maintaining robust TB control programs, capable of preserving diagnostic, preventive and treatment services.

Consequently, ensuring accurate diagnosis is a central priority in everyday clinical practice. However, as confirmed by the studies presented here and conducted within the PTBnet network, currently available immune-based assays, such as the tuberculin skin test and interferon-gamma release assays, remain insufficiently sensitive, particularly in young or immunocompromised children. Moreover, and crucially, they do not discriminate between infection and active disease.

This limitation must be considered in light of the fact that, as extensively discussed, establishing a diagnosis in children based on clinical, microbiological, and radiologic criteria is inherently more challenging than in adults.

Building upon these premises, an integrated immunologic and metabolomic approach to pediatric TB diagnostics was explored. The observed trends, higher HBHA responsiveness in latent or milder disease and distinct metabolomic signatures separating active from latent TB, indicate that integrating immunological and

metabolomic tools can enhance diagnostic discrimination between active TB and LTBI.

These results should be regarded as preliminary, and the identified trends require validation through forthcoming analyses by incorporating additional samples already collected from our study population and, where feasible, by assessing larger external cohorts. Nevertheless, they offer a proof of concept for integrating immunological and metabolomic modalities within pediatric TB diagnostics, pointing toward a multidimensional biomarker approach that could enhance diagnostic accuracy and thereby improve the clinical management of tuberculosis in children.

In conclusion, in childhood, the diagnostic gap, the difference between estimated and detected cases, remains particularly wide, reflecting the absence of effective diagnostic strategies for this age group. Pediatric TB is not a scaled-down version of the adult disease, but a distinct clinical entity that demands dedicated research and tailored diagnostic approaches. There is an urgent need for diagnostic tools tailored to children that are both sensitive and specific, ideally using easily obtainable specimens, in order to optimize individual care, support prevention programs, and thereby narrow the diagnostic gap.

In this context, intensified research and innovation to develop new diagnostics are one of the three pillars of WHO End TB Strategy and must be decisively strengthened if we are to approach its ambitious 2035 targets (95% reduction in TB mortality and a 90% reduction in incidence compared with 2015 levels), and, ultimately, move closer to ending a preventable and curable disease that still claims far too many lives. Addressing the specific challenges of pediatric TB is a crucial step on that path.

CHAPTER 6

Other research or academic items

This chapter presents a collection of papers from other research projects, as well as academic activities carried out during the PhD program (2022–25).

7.1. Full papers from other research projects

- 1) **Scarano SM**, Bruzzese E, Poeta M, Del Bene M, Guarino A, Lo Vecchio A. Dalbavancin for Acute Bacterial Skin and Skin Structure Infections in Pediatrics: Insights from Continuation Therapy Experience. *Antibiotics (Basel)*. 2024 Apr 4;13(4):327. doi: 10.3390/antibiotics13040327. PMID: 38667003; PMCID: PMC11047506.
- 2) Lo Vecchio A*, **Scarano SM***, Pierri L, Salerno M, Discepolo V, Giannattasio A, Buonsenso D, Farina AM, Catzola A, Poeta M, Nunziata F, Bruzzese E, Guarino A. High Cardiac Troponin Levels in Infants with Acute SARS-CoV-2 Infection: A Prospective Comparative Study. *J Pediatr*. 2024 Mar;266:113876. doi: 10.1016/j.jpeds.2023.113876. Epub 2023 Dec 21. PMID: 38135032.
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- 7) Poeta M, Nunziata F, Del Bene M, Morlino F, Salatto A, **Scarano SM**, Cioffi V, Amitrano M, Bruzzese E, Guarino A, Lo Vecchio A. Diarrhea Is a Hallmark of Inflammation in Pediatric COVID-19. *Viruses*. 2022 Dec 6;14(12):2723. doi: 10.3390/v14122723.

7.2 Conference abstract and presentations

- Central nervous system tuberculosis threat in children: a case series. Alfonso Maria Farina, **Sara Maria Scarano**, Valerio Colecchia, Maria Francesca Manca, Rita Nunziata, Giangiuseppe Senatore, Eugenia Bruzzese, Alfredo Guarino, Andrea Lo Vecchio.
43rd Annual Meeting of the European Society for Paediatric Infectious Diseases (ESPID), 26–30 May 2025, Bucharest, Romania.
- Bacillus Calmette-Guérin vaccine adverse reaction: a case report. Rita Nunziata, Alfonso Maria Farina, Giangiuseppe Senatore, **Sara Maria Scarano**, Eugenia Bruzzese, Alfredo Guarino, Andrea Lo Vecchio.

43rd Annual Meeting of the European Society for Paediatric Infectious Diseases (ESPID), 26–30 May 2025, Bucharest, Romania.

- Impact of Taurolidine prophylaxis on Central-Line associated bloodstream infections in children on long-term parenteral nutrition: a prospective cohort study. Rita Nunziata, **Sara Maria Scarano**, Alessia Morlando, Alfredo Guarino, Maria Immacolata Spagnuolo, Andrea Lo Vecchio.

42nd Annual Meeting of the European Society for Paediatric Infectious Diseases (ESPID), 20–24 May 2024, Copenhagen, Denmark. (*Poster presentation*)

- Severe hypertransaminasemia in children with tuberculosis treated with rifampicin/isoniazid/pyrazinamide fixed-dose therapy: a case-control study. **Sara Maria Scarano**, Marco Poeta, Valerio Colecchia, Margherita Del Bene, Andrea Lo Vecchio, Alfredo Guarino. ESPGHAN 56th Annual Meeting, 15–18 May 2024, Milan, Italy.

- Andrea Lo Vecchio, **Sara Maria Scarano**, Pietro Buono, Alessandro Scoppa, Chiara Amato, Valerio Colecchia, Alfredo Guarino. Co-administration of vaccines against Rotavirus and group B Meningococcus is associated with an increased Rotavirus vaccination coverage: a 5-year retrospective population study. 41st Annual Meeting of the European Society for Paediatric Infectious Diseases (ESPID), 8–12 May 2023, Lisbon, Portugal.

- **Sara Maria Scarano**, Pietro Buono, Alessandro Scoppa, Alfonso Maria Farina, Rita Nunziata, Alfredo Guarino, Andrea Lo Vecchio, Rotavirus immunization coverage and timing of administration: a regional prospective cohort study in Southern Italy.

41st Annual Meeting of the European Society for Paediatric Infectious Diseases (ESPID), 8–12 May 2023, Lisbon, Portugal.

- Federica Pagano, Benedetta Bocchini, Martina Mancuso, **Sara Maria Scarano**, Francesco Nunziata, Marco Poeta, Piera Abate, Andrea Lo Vecchio, Eugenia

Bruzzese, Roberto Della Casa. Multiple causes of Bell Paralysis in a toddler: which one is to treat?

41st Annual Meeting of the European Society for Paediatric Infectious Diseases (ESPID), 8–12 May 2023, Lisbon, Portugal.

- Cristina Moracas, Francesco Nunziata, Marco Poeta, **Sara Maria Scarano**, Margherita Del Bene, Marianna Alagia, Alfredo Guarino, Eugenia Bruzzese, Andrea Lo Vecchio. Multisystem Inflammatory Syndrome in children with acute Influenza A infection resulted negative for SARS-CoV.2 infection. *41st Annual Meeting of the European Society for Paediatric Infectious Diseases (ESPID)*, 8–12 May 2023, Lisbon, Portugal.
- Liana Punzi, Fabio Castellano, **Sara Maria Scarano**, Margherita Del Bene, Cristina Moracas, Davide Cagno, Martina Mancuso, Marco Poeta, Francesco Nunziata, Andrea Lo Vecchio, Eugenia Bruzzese. Kaposi sarcoma in pediatric HIV patient: case report.
41st Annual Meeting of the European Society for Paediatric Infectious Diseases (ESPID), 8–12 May 2023, Lisbon, Portugal.

7.3 Conference attendance

- 43rd Annual Meeting of the European Society for Paediatric Infectious Diseases (ESPID), 26–30 May 2025, Bucharest, Romania.
- XIX Congresso Nazionale Società Italiana di Infettivologia Pediatrica (SITIP) 8–20 March 2025, Naples, Italy.
- 42nd Annual Meeting of the European Society for Paediatric Infectious Diseases (ESPID), 20–24 May 2024, Copenhagen, Denmark.
- Stop TB Italia – Non è un paese per vecchi, VI edizione – 29 May 2024, Naples, Italy.

- 41st Annual Meeting of the European Society for Paediatric Infectious Diseases (ESPID), 8–12 May 2023, Lisbon, Portugal.