IP-10 for the diagnosis and treatment monitoring of tuberculosis in children.

Anna O. Komorowska-Piotrowska[1], Agnieszka M. Strzelak[1], Agnieszka Borowa[2], Maria Krasińska[2], Wojciech Feleszko[1], and Marek Kulus[1]

[1]Warszawski Uniwersytet Medyczny
[2]Instytut Gruzlicy i Chorob Pluc

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Abstract

Purpose: To determine the utility of interferon-gamma-inducible protein 10 (IP-10) for identifying active tuberculosis (TB) and latent TB infection (LTBI) in children in BCG-vaccinated population, establish its diagnostic performance characteristics, and evaluate changes in IP-10 level during anti-TB-chemotherapy. *Methods:* Concentrations of IP-10 and IFN-γ were measured in QuantiFERON-TB Gold (QFT) supernatants in children with suspected TB or due to a recent TB contact. A total of 225 children were investigated: 33 with active TB, 48 with LTBI, 83 TB contacts, 20 with suspected TB but other final diagnosis, and 41 controls. In 60 children cytokine responses were evaluated on follow-up visit after 2 months of anti-TB-treatment. *Results:* IP-10 expression was significantly higher in infected children (active TB and LTBI cases) than in uninfected individuals. IP-10 proved effective in identifying TB infection at its optimal cut-off (>1084.5 pg/ml), but was incapable of differentiating between children with active TB and LTBI. Combining IP-10 and IFN-γ increased QFT sensitivity. IP-10 but not IFN-γ decreased significantly during anti-TB-treatment in children with active TB ($p = 0.003$). *Conclusion:* IP-10 identifies TB infection and declines during anti-TB-chemotherapy in children. Incorporating IP-10 into new immunodiagnostic assays could improve TB diagnosis and allow treatment monitoring.

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Agnieszka M. Strzelak MD[1] (ORCID: 0000-0002-8313-1924), Anna O. Komorowska-Piotrowska MD[1] (ORCID: 0000-0001-5034-538X), Agnieszka Borowa MD[2], Maria Krasińska MD[2], Wojciech K. Feleszko PhD[1] (ORCID: 0000-0001-6613-2012), Marek K. Kulus PhD[1] (ORCID: 0000-0002-5360-4372).

[1] Department of Pediatric Pulmonology and Allergy, Medical University of Warsaw, 63A Zwirki i Wigury Street, 02-091 Warsaw, Poland

[2] Department of Lung Diseases and Tuberculosis for Children and Adolescents, Mazovian Center for Treatment of Lung Diseases and Tuberculosis, Reymonta 83/91 Street, 05-400 Otwock, Poland

Corresponding Author: Anna Komorowska-Piotrowska MD, Department of Pediatric Pulmonology and Allergy, Medical University of Warsaw, 63A Zwirki i Wigury Street, 02-091 Warsaw, Poland. Tel: (+)482231794-19; Fax: (+)48223179542;

E-mail: anna.komorowskapiotrowska@gmail.com

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ABSTRACT:

Purpose: To determine the utility of interferon-gamma-inducible protein 10 (IP-10) for identifying active tuberculosis (TB) and latent TB infection (LTBI) in children in BCG-vaccinated population, establish its diagnostic performance characteristics, and evaluate changes in IP-10 level during anti-TB-chemotherapy.

Methods: Concentrations of IP-10 and IFN-γ were measured in QuantiFERON-TB Gold (QFT) supernatants in children with suspected TB or due to a recent TB contact. A total of 225 children were investigated: 33 with active TB, 48 with LTBI, 83 TB contacts, 20 with suspected TB but other final diagnosis, and 41 controls. In 60 children cytokine responses were evaluated on follow-up visit after 2 months of anti-TB-treatment.

Results: IP-10 expression was significantly higher in infected children (active TB and LTBI cases) than in uninfected individuals. IP-10 proved effective in identifying TB infection at its optimal cut-off (>1084.5 pg/ml), but was incapable of differentiating between children with active TB and LTBI. Combining IP-10 and IFN-γ increased QFT sensitivity. IP-10 but not IFN-γ decreased significantly during anti-TB-treatment in children with active TB ($p = 0.003$).

Conclusion: IP-10 identifies TB infection and declines during anti-TB-chemotherapy in children. Incorporating IP-10 into new immunodiagnostic assays could improve TB diagnosis and allow treatment monitoring.

KEY WORDS:

Latent Tuberculosis, Chemokine CXCL10, *Mycobacterium tuberculosis,* LTBI, TB contact

INTRODUCTION:

Childhood tuberculosis (TB), caused by *Mycobacterium tuberculosis*(*Mtb* .), is still an urgent global health issue. The COVID-19 pandemic had a negative impact on TB disease in terms of access to diagnosis and treatment, therefore community transmission of infection is expected to increase in all age groups [1]. Epidemiological estimates of global childhood TB burden have been long underestimated. Firstly, childhood active TB (ATB) is very difficult to diagnose due to diverse clinical presentations, difficulties in obtaining specimens for microbiological evaluation, and paucibacillary disease [2,3]. Secondly, the diagnostic work-up for latent tuberculous infection (LTBI) lacks the gold standard and routinely used immunological tests, namely tuberculin skin test (TST) and Interferon gamma (IFN-γ) release assays (IGRAs), have considerable limitations in pediatric population.

TST shows cross-reactivity with bacillus Calmette-Guérin (BCG) and majority of non-tuberculous mycobacteria (NTMs) and has therefore low specificity in BCG vaccinated and NTM infected children[4,5]. Commercially available IGRAs, ELISA-based QuantiFERON –TB Gold In-Tube (QFT) and ELISPOT-based T SPOT.TB have comparable sensitivity and higher specificity than TST in identifying *Mtb.* Infection. However, they perform less reliable in human immune virus (HIV)-infected and young children, albeit with inconsistent results [6-9]. Neither test discriminates ATB from LTBI, nor are they licensed for ATB diagnosis. Yet, they are widely used to facilitate the diagnosis of ATB with sensitivity reaching 77-85%[10]. The new version of QFT test, QuantiFERON-TB Gold Plus, has so far exhibited similar properties to previous-generation IGRAs in children in both ATB and LTBI [10].

It is plausible that additional cytokine biomarkers may improve IGRAs' performance [11]. One of the most promising surrogate biomarker is the interferon-gamma-inducible protein 10 (IP-10), a chemokine expressed primarily by antigen-presenting cells upon stimulation by IFN-γ [12]. In the TB field, IP-10-based assays have emerged as alternatives to IGRAs with IP-10 being released in 100-fold higher concentrations than IFN-γ. IP-10 has comparable accuracy to IGRAs in identifying *Mtb.* infection in adults and children and may even perform better in HIV-infected and the young[11-19]. Additionally, IP-10 has been suggested a potential biomarker for treatment monitoring, but studies in pediatric populations are scarce [20,21].

The aim of the present study was to assess IP-10 responses in children with symptoms of ATB and in TB contact referrals in a low TB-incidence country where BCG vaccination is routinely administered at birth. We intended to examine the potential of IP-10 to differentiate between different stages of *Mtb* . infection, to

compare IP-10 with QFT and TST results, and to explore the influence of age and antituberculous treatment on IP-10 level.

METHODS:

Study population

To this prospective study we recruited children aged < 18 years admitted to the Children's Hospital, Medical University of Warsaw, Poland, and Mazovian Centre for Treatment of Lung Diseases and Tuberculosis, Otwock, Warsaw Agglomeration, Poland between May 2012 and September 2015. Eligible for participation were 1) children with clinical suspicion of TB and 2) children in recent contact with pulmonary TB (PTB). Exclusion criteria included immunosuppression, previous antituberculous treatment, and TST performed within the last 18 months to minimize the risk of boosting. Ethics approval for this study was provided by the Bioethical Committee of the Medical University of Warsaw and written informed consent was obtained from the legal guardian of each participant before enrollment.

Clinical and laboratory procedures

Demographics, history and clinical findings were recorded systematically using a case report form. All participants underwent peripheral blood testing including QFT. Children suspected of ATB and contact tracing referrals underwent standard diagnostic procedures encompassing TST, QFT, chest X-ray, and, if required, histological and bacteriological evaluation (microscopy, PCR, and culture). Not all participants received TST due to recurrent shortages of Purified Protein Derivative (PPD). For ethical reasons, we did not perform TST, chest X-ray and microbiological studies in the control group. All children with ATB received standard combined antituberculous treatment, children with LTBI and TB contacts aged < 5 years were offered preventive chemotherapy. A subgroup of patients underwent follow-up examination two months after treatment onset.

1. TST

Following an intradermal injection of 2 Tuberculin Units (0.1 ml) of RT 23 PPD (Statens Serum Institute, Copenhagen, Denmark) into the left lower arm, the transverse induration was recorded 48-72 h later. The TST was defined positive at [?] 10 mm induration.

2. QuantiFERON-TB GOLD In-tube[?]

QuantiFERON-TB GOLD In-tube[?] assay was performed according to the manufacturer's instructions, the concentration of IFN-γ was determined by using the QFT ELISA, and the results were defined using the manufacturer's software (Cellestis/Qiagen, Carnegie, Australia) [22].

3. IP-10 determination

IP-10 concentration was measured in the remaining supernatants from QFT tubes using the Quantikine ELISA Human IP-10 Immunoassay (R&D Systems Europe, Ltd., Abingdon, UK) according to the manufacturer's instructions. All samples were measured in duplicate. The plasma was diluted 1:10 and samples with values outside the upper limit of the standard curve were diluted and measured again. The lower limit of detection (sensitivity) was calculated to be 2.1pg/ml. IP-10 concentrations are expressed in pg/ml.

Classification of participants

Following clinical and diagnostic work-up, the patients were assigned to one of the five categories:

1. ATB diagnosed in symptomatic patients with microbiologically confirmed TB (mATB) (positive microscopy, PCR or culture result), or in patients fulfilling [?] two of the following three criteria: 1) clinical symptoms suggestive of ATB, 2) radiological findings consistent with ATB, and 3) a history of contact with PTB; all in conjunction with a positive clinical response to antituberculous treatment.

2. LTBI defined as a positive TST and/or IGRA result in asymptomatic children with a history of contact with PTB and a normal chest X-ray.

3. TB contacts – asymptomatic children with a history of contact with PTB, not fulfilling the criteria for ATB or LTBI.

4. non-TB – children with clinical suspicion of TB, but with other final diagnosis and a positive response to specific treatment.

5. controls – asymptomatic children after careful exclusion of TB contact and inflammatory comorbidities, and with negative IGRA result.

For simplicity reasons, the term "*Mtb.*- infected" refers to children with LTBI and ATB combined together and "*Mtb.*- uninfected" to TB contacts, non-TB group, and controls combined together.

Statistical analysis

Data were analyzed using STATISTICA 13.1 (SatSoft, Kraków, Poland) and Medcalc 19.1 (MedCalc Software, Ostend, Belgium). Figures were prepared in GraphPad Prism version 8 (GraphPad Software, San Diego, CA). Data were presented as medians and interquartile ranges (IQR). We performed chi-square test or Fisher's exact test for qualitative variables, and Kruskall-Wallis test or Mann Whitney test for quantitative variables. Changes in IFN-γ and IP-10 concentrations during treatment were assessed using Wilcoxon signed rank test. The Spearman's rank test was applied to assess biomarker correlation. The diagnostic performance of IP-10 was assessed using the receiver operating characteristic curve (ROC) analysis and the area under the curve (AUC). The highest values of the Youden's Index were used for selecting the cut-off values. The TB antigen and mitogen control concentrations of IP-10 were background-corrected by subtracting the concentration in the negative control tube. All tests were two-sided, and a p value of <0.05 was considered significant.

RESULTS:

Study participant characteristics

In total, 263 children were eligible for the study; 19 were excluded due to insufficient blood samples, 2 due to improper sample handling, 2 due to insufficient data, 10 due to technical reasons, and 5 children were excluded because the index case was diagnosed with NTMs and not*Mtb.* infection. A total of 225 children were included in the final analysis: 33 with ATB, 48 with LTBI, 83 TB contacts, 20 with a non-TB disease, and 41 controls. Patient's characteristics is shown in Table 1. ATB patients were older than other children ($p < 0.05$), and TB contacts were younger than *Mtb.*- infected children and non-TB group ($p < 0.01$). No other significant demographic differences were noted across TB classification groups. More than 96% of participants were BCG-vaccinated. Of 33 ATB patients, 9 (27.3%) had a positive culture result, 13 (39.4%) were admitted with a clinical suspicion of TB and 20 (60.6%) due to the recent TB contact. All ATB patients achieved the resolution of symptoms on antituberculous treatment. The non-TB children were diagnosed with pneumonia (n5), respiratory tract infections (n6), other respiratory diseases (n4), and "miscellanea" (n5).

IP-10 responses in Mtb.-infected and uninfected children

IP-10 responses in *Mtb* . antigen-stimulated, unstimulated, and mitogen stimulated supernatants are presented in Table 2. Upon*Mtb.* antigen stimulation the expression of IP-10 augmented significantly only in *Mtb.*- infected children ($p < 0.0001$).

Mtb. antigen-stimulated IP-10 responses were much higher in*Mtb.*- infected than in *Mtb.* -uninfected children ($p < 0.0001$). We then compared IP-10 levels between the subgroups of infected and uninfected children (ATB and LTBI vs TB contacts, non-TB, and controls separately) and the differences in IP-10 levels were still significant ($p < 0.0001$ for all pairwise comparisons) (Figure 1). While IP-10 level was higher in ATB than in LTBI group ($p = 0.047$), no differences were demonstrated among*Mtb.* -uninfected groups ($p > 0.05$).

IP-10 identifies Mtb. infection regardless of reason for admission

To reflect daily pediatric clinical practice, we performed a subgroup analysis regarding the reason for hospital admission (Figure 2). In children with clinical suspicion of TB, IP-10 concentration was significantly higher in ATB group than in non-TB group ($p < 0.0001$). Similarly, in contact tracing referrals, *Mtb.*- infected children expressed higher levels of IP-10 than TB contacts ($p < 0.0001$), also when ATB and LTBI groups were compared separately ($p < 0.0001$ for both comparisons).

IP-10 responses according to TST, QFT and microbiological tests results

Assuming that Mtb.-infected children constitute a heterogenous group of patients in terms of TST, QFT, and microbiological confirmation, we compared children with different test results configuration for IP-10 expression. We found significant differences within LTBI group with higher IP-10 levels in children with TST^+QFT^+ LTBI than in those with discordant TST and QFT results (TST^-QFT^+ and TST^+QFT^-; $p = 0.009$ and $p < 0.0001$, respectively). No differences were observed between children with discordance ($p > 0.05$). In ATB group, there were no differences in IP-10 levels between children with and without microbiological confirmation, or with and without positive culture result ($p = 0.79$ and $p = 0.53$, respectively).

ΙΠ-10 ανδ ΙΦΝ-γ λεελ αρε νοτ ςορρελατεδ ωιτη αγε

Mtb. antigen-stimulated concentrations of IP-10 and IFN-γ differed significantly among four different age groups (0-2 yrs; 2-5 yrs; 5-10 yrs, and >10 yrs) ($p < 0.001$ and $p = 0.04$, respectively) and between children aged < 5 years and older ($p < 0.001$ and $p = 0.005$, respectively). However, the correlation between biomarker level upon *Mtb.* antigen stimulation and age was weak for IP-10 and negligible for IFN-γ ($r_s = 0.32$; $p = 0.000001$ and $r_s = 0.16$; $p = 0.014$, respectively).

IP-10 level decreases during antituberculous treatment

After two months of antituberculous therapy (median 69; IQR:64 -78.5 days from treatment onset) IP-10 and IFN-γ levels in QFT tubes were measured in 25 children with ATB, 29 with LTBI, and 6 TB contacts. We noticed a significant decrease in the IP-10 but not IFN-γ level in the whole group after two months of treatment ($p = 0.0013$ and $p = 0.6$, respectively) (Figure 3A and 3C). This decrease resulted from a significant decline in the IP-10 concentration in the ATB group (Figure 3B and 3D). No significant decline in IP-10 level was observed in LTBI group ($p = 0.09$).

After the initial phase of chemotherapy IP-10 responses still differed significantly between *Mtb.*- infected and uninfected children (median 1324.4 pg/ml; IQR: 210.9 - 10134.9 pg/ml vs median 111.5 pg/ml; IQR: 0 - 164.6 pg/ml, $p = 0.008$). IP-10 was significantly higher in children with ATB (median 4139.7 pg/ml; IQR: 1090- 10464.6 pg/ml) than in TB contacts (median 111.5 pg/ml; IQR: 0 - 164.6 pg/ml) ($p = 0.0007$). However, no significant differences were found between TB contacts and children with LTBI (median 658.2 pg/ml; IQR: 36.8 - 5295.4 pg/ml) ($p > 0.05$). On follow-up visit we still observed a significant difference in IP-10 level between ATB and LTBI groups ($p = 0.015$). Of note, no discrepancies in IFN-γ concentrations were found between children with ATB, LTBI, and TB contacts (data not shown).

Diagnostic performance of IP-10

Only data from children with microbiologically confirmed ATB, TST^+QFT^+ LTBI, TST^-QFT^- TB contact, TST^-QFT^- non-TB, and QFT^- controls were included in the ROC analysis. For the assessment of IP-10 accuracy in identifying ATB and LTBI, data from children with ATB and LTBI were compared with the control group. IP-10 showed very good properties for discriminating *Mtb.*- infected children, children with ATB, and children with LTBI from controls. However, IP-10 could not discriminate between ATB and LTBI groups. Additionally, ROC analysis was performed separately for children admitted due to clinical suspicion of TB and for contact tracing referrals. In both settings we observed good performance of IP-10 in identifying *Mtb.* -infected children. At its optimal cut-off values IP-10 showed very good performance in each setting (AUC > 0.9) except ATB *versus* LTBI (Table 3), and outperformed IFN-γ (data not shown).

We then evaluated the properties of the optimal cut-off point of 1084.5 pg/ml for identifying *Mtb.* infection (ATB and LTBI combined vs control group). At this cut-off IP-10 correctly classified 84.9% of the partic-

ipants, 93.3% among microbiologically confirmed ATB group, and showed 88.5% positive predictive value and 83.5% negative predictive value. In children with ATB, IP-10 correctly classified 78.8% of patients and performed better than QFT but poorer than TST (69.7%. and 93.3%, respectively). One child with indeterminate QFT result was correctly classified by both TST and IP-10. Combining IP-10 and QFT results increased sensitivity to 84.5%.

DISCUSSION:

The present study indicates the potential of IP-10 to detect *Mtb.*infection in children in a low TB incidence country where BCG vaccination is routinely administrated at birth. We demonstrate that IP-10 level is not correlated with age and, for the first time, report a decrease in the IP-10 level on antituberculous treatment in children.

In agreement with previous studies, we showed that IP-10 can distinguish*Mtb.* -infected from uninfected children[17-19,21,23-26]. Unlike some other reports, we observed an increased *Mtb.* antigen-stimulated expression of IP-10 only in the infected children [21,25]. While demonstrated significant differences in IP-10 level between *Mtb.*- infected and uninfected contact tracing referrals have been already reported by others [15,19,21,24,25], IP-10 has not been vastly investigated in symptomatic children with a clinical suspicion of ATB. As far as we know, this study is the first to show that IP-10 can identify *Mtb.* infection in symptomatic children under diagnosis for TB in a low-endemic setting. Previous studies by Petrone et al. and Sudbury et al. were performed in high-incidence countries[27,28]. Petrone et al. reported contradictory results to our study but employed unstimulated plasma, which may have accounted for the observed differences [27]. In line with our report, Sudbury et al. noted significantly higher IP-10 level in ATB children than in symptomatic children with other final diagnosis[28]. However, the number of ATB patients was low (n=5 as compared to n=33 in this report).

Although *Mtb.* antigen-stimulated IP-10 level was higher in children with ATB than in LTBI group ($p = 0.047$), no power of IP-10 to discriminate between ATB and LTBI has been demonstrated in ROC analysis. Our findings stay in line with the majority of previous reports in adults and children [17,20,29,30].

We observed a good agreement between IP-10 and both TST and QFT (85.4% and 87.7% respectively; =0.7; p=0.0000; data not shown). Moreover, combining IP-10 and IFN-γ results improved QFT sensitivity in patients with ATB, in whom IGRAs' performance remains suboptimal. The benefits of combining biomarker approach in childhood TB have been already documented [15-17,23,26,30]. However, several studies conducted in a high endemic setting and in children with ATB yielded conflicting results warranting further research in this field[18,31,32].

On par with most evidence in children, we did not demonstrate a relevant correlation of IP-10 with age [18,23,25,30]. Additionally, in line with a previous study from our center, we did not observe compromised IFN-γ responses in younger children[9].

Furthermore, the present study is, as far as we know, the first to report a decrease in the IP-10 level on anti-TB treatment in children. While a decline in plasma IP-10 level after successful anti-TB treatment has been demonstrated in adults, it has not been vastly investigated in children [20,21,33-36]. Nausch et al. analyzed a spectrum of cytokines before and during anti-TB treatment and reported a significant reduction only in the expression of IFN-γ with no change in the IP-10 level [21]. In contrast, we observed a significant decline in the IP-10 concentration with no decrease in the IFN-γ level. This decline in the IP-10 expression was detected only in children with ATB, who had the highest concentrations at baseline. We assume that differences in time to follow-up visit (median 69 days in our study versus 90 days in the other report) could explain the discrepancies related to IFN-γ but not IP-10. Our results support the previous study by Wergeland et al., who demonstrated a decrease in IP-10 level in adult patients with ATB already after 6-12 weeks of treatment[20].

Another strength of the present study is the control group consisting of healthy children with a population risk of TB infection. Numerous studies have included TB contacts, children with respiratory tract infections, or

healthy adults in the control group[17,19,21,24,25,27,30]. We consider our approach reasonable for several reasons. First, *Mtb.* infection cannot be definitively excluded in children recently exposed to TB. Second, IP-10 can be induced during diverse inflammatory diseases including infections[37,38]. Third, direct comparison of IP-10 level solely between children provides relevant information on its' expression in this age group. Taken together, including healthy children in the control group might have limited the risk of bias in the present report.

The main limitation of our study is the relatively small sample size. This is not uncommon in studies conducted in children in TB low-burden areas and has been previously reported [19,30]. Furthermore, our results might be biased by potential false classification of participants. Similar to others, we applied stringent criteria for ATB diagnosis to limit the risk of misclassification[19,21,25]. The rate of culture-positive cases reflected other observations in children, and no significant differences in IP-10 or IFN-γ level (data not shown) were demonstrated between ATB children with and without a positive culture result [39]. Therefore, we assume that the classification of ATB patients was correct, however, the possibility of misclassification cannot be truly excluded.

Furthermore, LTBI group in the present study comprises children with either positive TST or QFT result. Since the TST false-positivity rate does not exceed 8.5 % in individuals vaccinated in infancy and only 2 participants with TST^{-}/QFT^{+}discordance presented with QFT results relatively close to the cut-off (data not shown), we believe this cannot account for a large proportion of discordant LTBI patients in our report [4]. Similar to others, we observed higher IP-10 levels in children with $TST^{+}QFT^{+}$ LTBI than in children with discordance [16,19]. Interestingly, contrary to Petrucci et al., no differences were noted between children with different discordant results constellation. Since children with discordance constitute a significant management problem in pediatrics, this is an area that merits further research on fluctuations of TB biomarkers during different stages of *Mtb.* infection.

Applying QFT negativity as a criterion for *Mtb.*- uninfected groups might have caused selection bias and rendered the comparison of the specificity of IP-10 and QFT impossible. Furthermore, we neither assessed the severity of TB, nor were children with the most severe forms of disease included. Additionally, children with ATB were significantly older than children in the other groups. Therefore, IP-10 performance in children with severe TB and the youngest children with ATB may differ from that presented here. Routine testing for HIV was not performed as part of this study, but HIV tests results were recorded if available and no cases were reported. Notably, HIV incidence in Poland is low (<1/100 000) with marginal TB-HIV coexistence[40]. Nonetheless, the performance of IP-10 in HIV infected children in our setting remains uncertain.

In conclusion, our data demonstrate that *Mtb.*- specific IP-10 measurement has a potential as a diagnostic biomarker of childhood TB. In particular, IP-10 seems to be a valid surrogate marker to IFN-γ in the assays based on the QFT platform. Our data show that IP-10 alone does not allow the distinction between ATB and LTBI. This is the first study with a clinical approach to TB diagnosis in children that shows good performance of IP-10 in identifying *Mtb* . infection in TB contact referrals and children with clinical suspicion of TB in a low TB-endemic country. Additionally, this is the first report demonstrating a significant decline of the IP-10 level during anti-TB chemotherapy in children. Collectively, these findings suggest IP-10 has the potential to become a TB marker in pediatric population and may be used in treatment monitoring.

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Availability of data and materials: The datasets used and analysed during the current study are available from the corresponding author on reasonable request.

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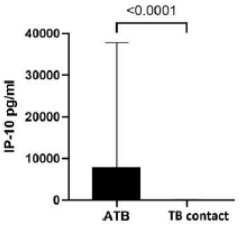

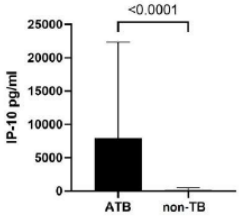

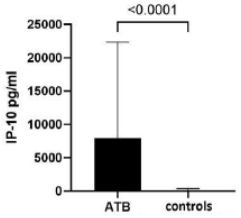

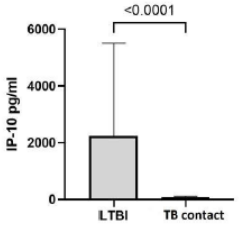

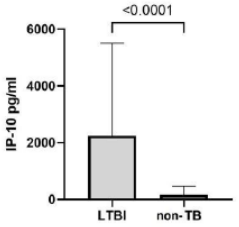

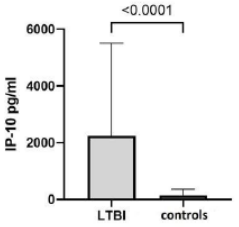

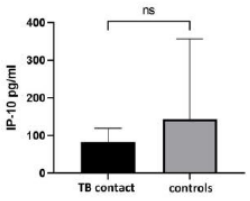

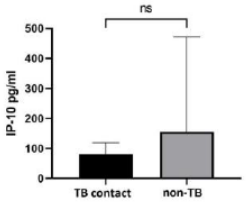

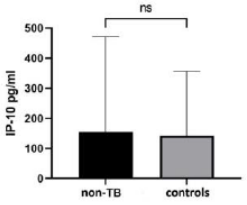

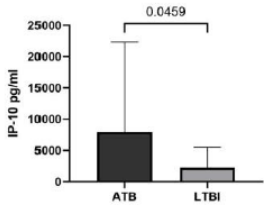

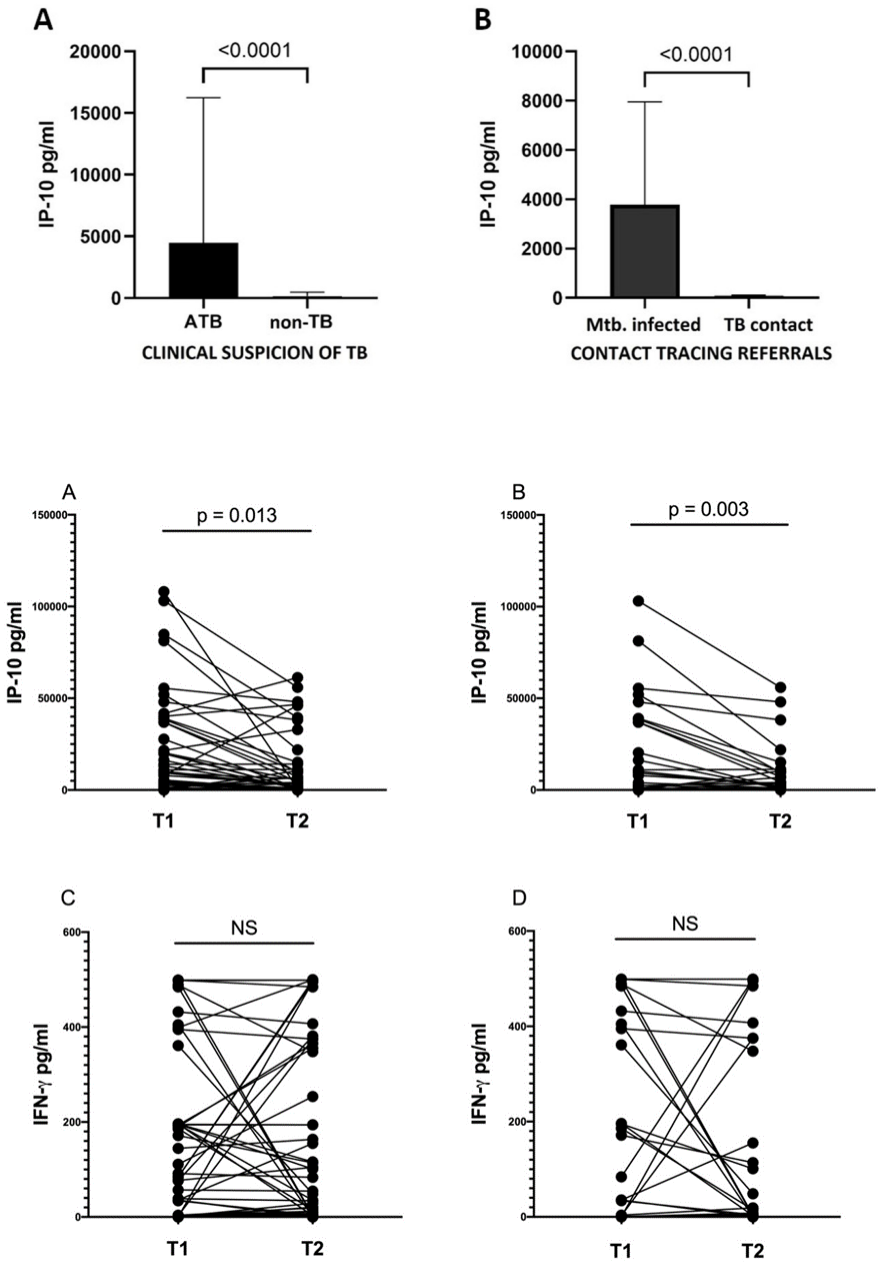

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