Title: Diagnosing TB infection in children: analysis of discordances using in vitro tests and tuberculin skin test

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ABSTRACT

Objectives: To study the performance of the IFN-γ tests (QuantiFERON-TB-Gold In Tube [QFN-G-IT] and T-SPOT.TB) and the tuberculin skin test (TST) in diagnosing tuberculosis infection in children, and to analyse discordant results.

Patients and Methods: A prospective study including 98 children from contact-tracing studies; and 68 children with TST≥5mm recruited during public health screenings.

Results: Positive IFN-γ tests results were associated with risk of exposure (p<0.0001). T-SPOT.TB was positive in 11/14 cases with active TB (78.6%) and QFN-G-IT in 9/14 (64.3%). In 6 of 12 children non-BCG-vaccinated, with a TST induration between 5 and 9 mm and both IFN-γ tests negatives, the detection of sensitised T cells against Mycobacterium avium was positive. In concordant IFN-γ tests results, a positive correlation was found (p=0.0001) between the number of responding cells and the amount of IFN-γ released; however, in discordant IFN-γ tests results this correlation was negative (p=0.371): an increase in the number of spot forming cells correlated with a decrease in the amount of IFN-γ released.

Conclusions: The use of IFN-γ tests is helpful for the diagnosis of TB infection, avoiding cross-reactions with BCG immunisation and NTM infections. The analysis of highly discordant results requires further investigation to elucidate possible clinical implications.

KEYWORDS
Agreement; Children; Interferon-gamma release assays; Non-tuberculous mycobacteria sensitins; Tuberculin skin test; Tuberculosis.
In 2007 the estimated global incidence of tuberculosis (TB) cases was 9.27 million. Approximately 11% of these cases were children. In the developed world the estimated proportion of children with TB is around 3-6%, but in developing countries this percentage can reach 15-20%, with an approximate mortality of 30% [1]. Latent tuberculosis infection (LTBI) treatment is an essential strategy to eliminate TB [2], though to achieve any epidemiological impact this strategy must target groups with high risk of infection and development of the disease if they get infected. Children merit special consideration since they can develop the disease very quickly after primary infection, with the most severe forms prevailing in younger children [3].

The advantages of the techniques based on the detection of gamma interferon (IFN-\(\gamma\)) secreted by effector T cells stimulated with specific Mycobacterium tuberculosis antigens to diagnose LTBI, over the tuberculin skin test (TST) are essentially the lack of cross-reactivity with vaccinal Mycobacterium bovis Bacillus Calmette-Guérin strains (BCG), and non tuberculous mycobacteria (NTM), and the absence of booster effect [4, 5]. These antigens are the early secretory antigenic target 6 (ESAT-6) and culture filtrate protein 10 (CFP-10) encoded in the region of difference (RD) 1, and TB7.7 encoded in RD11, absent in all BCG strains and in the majority of NTM. Basically, two commercialized in vitro assays based on this technology are currently available: QuantiFERON-TB GOLD In Tube (QFN-G-IT) (Cellestis, Carnegie, Australia) and T-SPOT.TB (Oxford Immunotec, Oxford, UK). Studies in adults have shown a high sensitivity and specificity for TB diagnosis [5-8]. In a recent systematic review [5], using active TB as a surrogate for LTBI, sensitivities values were 70% (95% confidence intervals [CI]:63-78) for QFN-G-IT and 90% (95% CI:86-92%) for T-SPOT.TB; and specificity: 96% (95%CI: 94-98%) for QFN-G-IT, and 93% (95% CI:86-100%) for T-SPOT.TB.

The objectives of this study were: to compare QFN-G-IT and T-SPOT.TB results with those obtained by TST for the diagnosis of TB infection in children in a referral clinical centre for TB control and to analyse their concordant or discordant results.

**MATERIALS AND METHODS**

**Study design.** Prospective study in children (≤15 years old) who attended the “Unitat Clínica de Prevenció i Control de la Tuberculosis” in Barcelona (Spain) between September 2005 and September 2007. This study was approved by the Ethics Committees of Fundació Jordi Gol i Gurina and of the Hospital Universitari Germans Trias i Pujol (Spain). Parents were asked to sign an informed consent form.
Children were divided in two groups: a contact group (CG) including children studied due to a close contact with a smear positive active TB patient diagnosed within the last 15 days; and a screening group (SG): healthy children with a positive TST result detected during an epidemiological screening at school or by their paediatrician.

Data were collected by means of a structured interview. A clinical examination, TST result, chest X-rays and both IFN-\(\gamma\) tests were performed. The presence of a BCG scar was recorded. Children were excluded if they had a previous history of any TB treatment.

The risk of infection was classified into three groups: a) High: same household and different household, but with a daily contact of 6 hours or more with the contagious index case; b) Medium: non-daily contact with the contagious index case, minimum once weekly; c) No risk known: children from SG without any TB index case known.

A blood drawn was performed within the 5 days after the TST performance. The study was double-blinded: the clinical diagnosis of TB was made without knowing the IFN-\(\gamma\) test results, and the researchers in the laboratory did not know the clinical data prior to the performance of the tests.

**TST.** The test was performed with 2 units of purified protein derivative RT23 [1]. TST was considered positive when the induration was \(\geq 5\) mm in contacts and in children with abnormal chest radiographs consistent with active TB; and \(\geq 10\) mm for children in the SG, irrespective of BCG immunisation.

**Active TB diagnosis.** We followed national guidelines for the diagnosis of the active TB cases [9, 10]. A TB case was considered as a child with an isolation of *M. tuberculosis* from clinical specimens, or a child with presence of symptoms, signs and/or radiological images compatible with TB (when Chest-X-Ray was doubtful CT thoracic scan was performed); and/or TST positive (as defined previously), and child who clinically responded to anti-tuberculous chemotherapy. The fact of being a close contact of a bacillary TB case was used as a diagnostic support.

**T-SPOT.TB.** Specific mononuclear cells isolated from peripheral blood (PBMCs) were stimulated with ESAT-6 and CFP-10 separately, according to manufacturer’s recommendations. Positive, negative and indeterminate results were strictly interpreted
following manufacturer’s instructions. Non-stimulated cells were washed with RPMI medium (Invitrogen, Auckland, NZ) and resuspended in freeze medium (80% RPMI and 20% foetal bovine serum [PAA Laboratories GmbH, Pashing, Austria]) adding, drop wise, 10% of DMSO (Merck, Darmstadt, Germany) and then frozen at -80°C. We considered the sum of spot forming cells (SFCs) obtained after ESAT-6 and CFP-10 stimulation as an overall RD1 response [11].

**Ex vivo detection of T-cell sensitised against *M. avium* sensitin.** To investigate the influence of NTM infections on non-BCG-vaccinated children, with a TST induration between 5 to 9 mm and both IFN-γ tests with a negative results, we performed an ex vivo ELISPOT, stimulating the cells with *Mycobacterium avium* sensitin. Cells were thawed and re-suspended in RPMI medium. Then, cells were washed, re-suspended and stimulated with medium alone, phytohaemagglutinin and *M. avium* sensitin (10 μg/mL) (Statens Serum Institute, Copenhagen, Denmark) as previously described [12]. Sensitised cells were detected by ELISPOT. The interpretation of the results followed the same criteria as that for detecting ESAT-6 and CFP-10 immunoresponse.

**QFN-G-IT.** The test detects IFN-γ released from T cells stimulated with the specific antigens in whole blood. QFT-G-IT incorporates specific antigens (ESAT-6, CFP-10, and TB7.7) inside the same blood collection tube. The test was performed and the results were interpreted according to the manufacturer’s instructions.

**Statistical methods.** The qualitative variables description is based on the calculation of the number and its percentage, and for quantitative variables, on calculation of the mean and the standard deviation (SD). The chi-squared test and two-tailed Fisher's exact test were used to compare qualitative variables. The odds ratios (OR) and its 95% confidence intervals (95%CI) were calculated. The associated variables with a value p<0.05 were analysed at a multivariate level by means of logistic regression. Non-parametric tests (Mann-Whitney, Kolmogorov-Smirnov, Kruskal-Wallis) were used to compare quantitative variables according to the categories of the group variable. Graphic analysis and Pearson correlation techniques (CC) were used to study the association. Cohen’s kappa coefficient (k) was used to analyse the concordance, its p value and standard error (according to Landis and Cock estimation). The area under the receiver operating characteristic (ROC) curve was calculated to compare the diagnostic performance of the TST, T-SPOT.TB and QFN-G-IT in the diagnosis of active TB. The data were analysed using SPSS (version 14.0; SPSS Inc., Chicago; IL, USA).
RESULTS

**Clinical performance.** A total of 166 children were included in the study, 84 (50.6 %) were female. The age (mean±SD) was 9.08±4.85 years. Ninety eight (59%) were contacts and 68 (41%) belonged to the SG. In 149 children (89.8%) TST was positive. This high percentage of TST positive results is due to the fact that all children included in the SG group were TST positive. The IFN-γ tests (either one or both) were positive in 72 children (43.4%; 95%CI=35.7-51.3): 54 contacts (55.1%; 95%CI=44.7-65.2) and 18 from the SG (26.5%; 95%CI=16.5-38.6). T-SPOT.TB was positive in 64 children (38.6%; 95%CI=31.1-46.4) and QFN-G-IT in 61 (36.7%; 95%CI=29.4-44.6) (Table 1). Treatment of LTBI was considered according to the TST result, consequently children who had positive TST and negative IFN-γ tests were treated and conventional follow-up and control was done.

All children considered not TB infected according to the TST result obtained a negative IFN-γ-based tests result. From the 20 non-BCG-vaccinated children from the SG, both IFN-γ tests were negative in 14 children with a TST induration between 5 and 9 mm. There were 48 BCG-vaccinated children in the SG: T-SPOT.TB was positive in 11/48 (22.9%) and QFN-G-IT in 9/48 (18.75%). In the 3 of them with TST induration between 5-9 mm both IFN-γ tests were negative. Therefore in the 45 children who had a positive TST (induration ≥10 mm), T-SPOT.TB was positive in 11/45 (24.4%), and QFN-G-IT in 9/45 (20%). Distribution of IFN-γ tests and TST results according to BCG and non-BCG-vaccinated status, and contact and screening groups, are shown in figures 1 and 2. No indeterminate results were detected by QFN-G-IT, but by T-SPOT.TB (1.8%) in 3 cases the test failed because the blood volume drawn was insufficient.

IFN-γ tests were in agreement in 146/166 children (Table 2). None of the variables that might have influenced the level of concordance between both tests was significantly associated with the outcome. There were no significant differences in age, gender or study group, between the children with concordant and discordant IFN-γ results (data not shown). IFN-γ tests were discordant in 20 children (12.04%). The 3 failed cases in the T-SPOT.TB belonged to the CG (3.06%) and among them there was a 3 year old patient with active TB. The overall agreement was 89.6% (κ=0.778) after excluding the failed cases.
**Variables related to the positivity of IFN-γ tests.** Variables significantly associated with IFN-γ test positivity are shown in Table 1. In the multivariate analysis a positive T-SPOT.TB was associated with the fact of being a contact (p<0.001) and having an abnormal chest X-ray and for the QFN-G-IT was to be a contact (p<0.001) and not to be BCG-vaccinated (p = 0.01) (Table 1).

In Table 3, the positivity of the IFN-γ tests according to the risk of exposure to an infectious source is shown. The probability of a positive IFN-γ test (OR=3.60; 95%CI=1.85-7.04) was significantly associated with an increasing risk of exposure, independent of age and gender in the multivariate analysis (p<0.001). In addition, in the multivariate analysis the main factors associated with a positive T-SPOT.TB in the CG were a daily contact over 6 hours (OR=3.5; 95%CI=1.1-12.1; p=0.03) and an exposure time over 30 days (OR=1.9; 95%CI=1.1-6.9; p=0.04). However, no significant associations were found for the QFN-G-IT.

**Clinical performance of the IFN-γ tests in active primary TB.** Fourteen cases were finally classified as active primary TB. In 4 cases a microbiological confirmation was possible (positive culture for *M. tuberculosis*: 3 in gastric aspirate samples and 1 in sputum sample). In 8 cases the children were from the CG and in 6 from the SG. T-SPOT.TB was positive in 11/14 cases (78.6%) and the QFN-G-IT in 9/14 (64.3%). Both IFN-γ tests were positive in 8 children (57.1%) and negative in 2 cases (21.4%). For 1 patient the T-SPOT.TB failed and the QFN-G-IT positive; and 3 had a negative QFN-G-IT and a positive T-SPOT.TB. On the other hand, the differences in the number of responding T cells after stimulation with the specific antigens in the comparison between children diagnosed with active TB and all children without disease was significant (p=0.01 for ESAT-6 and CFP-10, respectively; and p=0.009 for RD1), but differences in the IFN-γ released did not reach statistical significance (p=0.09). However, if we exclude from the analysis children who were not diagnosed of LTBI by IFN-γ tests (both T-SPOT.TB and QFN-G-IT negatives), then there are not statistical significant differences in the number of responding T cells and the amount of IFN-γ released after antigen stimulation between active and LTBI children (Table 4).

If we consider children diagnosed with active TB as truly infected, and children from CG with a TST <5 mm and children from SG with a TST<10mm as truly not infected, then we could assume that the sensitivity and specificity of the IFN-γ tests is 78.57% (11/14), and 100% (35/35), respectively.
**Agreement between IFN-γ tests and TST.** The agreement between TST and IFN-γ tests is high in non-BCG-vaccinated children (Table 5). Variables associated with discordant results between TST and IFN-γ tests in multivariate analysis were: belonging to the SG (adjusted OR=6.9; 95%CI=3.4–14.4; p<0.001), being vaccinated with BCG (adjusted OR=10.1; 95%CI=3.3–30.9; p<0.001), and a TST with induration between 5-9 mm (adjusted OR=10.4; 95%CI=3.5–31.1; p<0.001).

Among the 17 autochthonous children from Spain, non-BCG-vaccinated, with a TST result between 5-9 mm of induration and with negative IFN-γ tests, the detection of sensitised T cells against *M. avium* sensitin was performed in 12 cases. In 3 cases the test failed due not having a sufficient number of cells recovered. It was negative in 3 cases and in the remaining 6 it was positive.

**Relationship between number of sensitised T cells and the amount of IFN-γ released.** When both IFN-γ tests agreed, high SFCs counts by T-SPOT-TB also showed high amounts of released IFN-γ (measured by QFN-G-IT). However, this correlation is negative in those children with a discordant result, where an increase in SFCs correlates with a decrease in IFN-γ released. In this case, the amount of IFN-γ tends to plateau. At this point, few cells produce high quantities of IFN-γ (negative T-SPOT.TB and positive QFN-G-IT), whereas the total amount of IFN-γ decreases or remains constant despite an increase in the SFCs (positive T-SPOT.TB and negative QFN-G-IT) (Figure 3).

On the other hand, as the diameter of the TST induration increases there is an increase in the SFCs (CC=0.09; p<0.0001) and in the total amount of IFN-γ released (CC=0.03; p<0.01); similarly, as the number of SFCs increases there is also an increase in the IFN-γ released (CC=0.27; p<0.0001). However, the correlation between TST induration and the SFCs and the IFN-γ released varies depending on whether the IFN-γ tests agree or not. When both IFN-γ tests agree, as the diameter of the TST induration increases there is an increase of responding SFCs (CC=0.315; p<0.0001), the regression line slope being 2.986 (p<0.0001); and there is also an increase of the IFN-γ released (CC=0.167; p=0.045), with a regression line slope of 0.343 (p=0.046). When there is no agreement between the IFN-γ tests, there is no correlation between the TST and the SFCs produced (CC=0.065; p=0.786) being the slope of the line...
almost null 0.189 (p=0.910); neither with the amount of IFN-γ produced (CC=0.362; p=0.117), being the slope of the regression line 0.298 (p=0.069).

DISCUSSION
This study shows the results of IFN-γ tests measurements in children seen in a reference centre for the diagnosis of TB infection, and compares the techniques currently available. Although the specificity for active TB for both tests was 100%, T-SPOT.TB obtained more positive results than QFN-G-IT in all groups analysed.

Our results highlight the usefulness of the IFN-γ tests compared to TST in the diagnosis of LTBI in contacts, as an association was found with the increase in the risk of infection and the exposure. These data agree with findings in other studies that have investigated TB outbreaks and study contacts [4, 11, 13-18]. These results also show the usefulness of IFN-γ tests to diagnose LTBI in BCG-vaccinated children when they are screened as part of paediatric or epidemiological control.

We have found that both IFN-γ tests show sensitivity over 75% and specificity of 100% for the diagnosis of active TB. Liebeschuetz et al [19] reported a sensitivity of 83% for T-SPOT.TB in African children. Nicol et al [20] described T-SPOT.TB positive results in 70% of children with clinical TB. Detjen et al [21] found a sensitivity of 93% for both IFN-γ tests when evaluating children with active TB. In addition, Connell et al [22] also reported positive IFN-γ tests in the 9 children diagnosed with active TB. In contrast with our results, Kampmann et al [23] found better results for QFN-G-IT than for T-SPOT.TB in children with culture-confirmed TB. Even if IFN-γ tests have been developed to diagnose LTBI, an alternative approach to the evaluation of the sensitivity of the in vitro tests has been to test patients with active TB. Although patients with active TB are infected by definition with M. tuberculosis, they do not have a LTBI. In fact, active TB occurs when the host immune responses are unable to contain the latent infection. Therefore, it should be considered that the value of the IFN-γ assays in active TB diagnosis is limited. False negative results of both tests in active TB have been described previously [6, 24, 25]. In addition, it has been reported that young children with severe active TB can have a reduced number of lymphocytes or a reduced lymphocyte function that could affect the sensitivity of the IFN-γ tests. In fact, in our study, in 6 children aged less than 3 years old, the T-SPOT.TB, was negative in 3 cases, failed in 2, and was positive in only one case; and the QFN-G-IT was negative in 3, and positive in the remaining 3 cases. However, no very severe TB presentation
was diagnosed in children with both IFN-γ tests negative. Other factors also involved could be the release of anti-inflammatory cytokines by PBMC and the temporary depression of T cell responsiveness [26, 27].

On the other hand, we have observed in our study that the IFN-γ assays are not able to distinguish between LTBI and active TB. No significant differences were detected between infected and diseased children in the number of responding T cells and the amount of IFN-γ released after antigen stimulation. The absence of significant differences in the response between active TB and LTBI could be explained by the fact that pediatric infection is usually recent. Therefore, the response is still strong, being similar to the one obtained during active TB [28].

Indeterminate results can be due to different causes, though they are generally due to a failure of the positive control. These results have been associated with immunosuppression, young age (<5 years) and a negative TST [14, 19, 20, 29]. Interestingly new information from different studies suggests that the increased frequency of indeterminate results in young children reflects a performance characteristic of the in vitro tests rather than a responding impairment to specific antigens and PHA [17, 30, 31]. An important source of failed results in young children has been related to an inadequate PBMC separation as a consequence of insufficient blood taken (especially in very young children) [13, 17, 23], which is the case in the 3 children who had a failed T-SPOT.TB result in our study. From our point of view, this kind of results should be considered since in children (where blood drawn is not always easy) these problems are inherent to the in vitro tests. However, given that in the QFN-G-IT assays no T cells count are required, we can not assess the impact of this kind of failure in the performance of the test.

It is difficult to compare the agreement between IFN-γ tests and TST with the results obtained by other authors because in each case positivity cut-off needs to be taken into account. In published studies this threshold can vary greatly, from 5, 10 and up to 15 mm of induration as indicative of TB infection [15, 17, 19, 20, 29] and generally depends on population groups (contacts, level of risk of development of active TB) and specific guidelines of the country.

The variables associated with discordance between the TST and IFN-γ test measurements were BCG immunisation, belonging to SG, and a TST induration of 5 to
In the SG an induration ≤ 10 mm is most likely caused by a NTM (non-specific sensitisation). In fact, in our study we detected T cell sensitization against *M. avium* sensitins in 6 out of 9 (66.7%) of these children. The existence of NTM in Spain was shown by Bleiker [32], and recently our group has described its presence in Catalonia [33]. Also, Detjen et al [21] showed the specificity of the IFN-γ tests in infections caused by NTM, and other authors have also described low agreement between IFN-γ tests and positive TST [7, 34]. Our group, in a previous publication, reported that 47.6% (10/21) of children with TST positive and negative T-SPOT. *TB* had sensitised T cells against *M. avium* sensitins [12]. Given that *M. avium* sensitin is not totally specific, we cannot totally exclude the possibility that we are detecting, in some cases, a response of specific T cells against some *M. tuberculosis* antigens different from ESAT6, CFP.10 and TB7.7. In order to reduce as much as possible this possibility we have focused our study on unexposed children with 5 to 9 mm of TST induration. Based in the classical studies performed by Nyboe *et al* [35], the main guidelines in screening children population consider as a cut-off for *M. tuberculosis* infection a TST induration equal to or higher than 10 mm, in order to avoid false positive TST results induced by NTM immunisation [36]. Therefore, our results reinforce, in part, the guidelines [10] in that unnecessary chemoprophylaxis treatment in unexposed population could be avoided, and that IFN-γ based assays could help to confirm a positive TST result. Nevertheless, indurations higher than 15 mm [21] and 20 mm [37] have been reported in children with NTM infections.

The main limitation of our study is that 89.75% of patients included had a positive TST (i.e., all children from the SG). This fact could introduce a bias in the comparison between the TST and IFN-γ tests due to the low number of negative TST results. Nevertheless, despite this limitation, the results obtained are sufficiently consistent to draw some conclusions about their utility in the diagnosis of LTBI in a referral centre.

Both IFN-γ tests have high agreement in our study. Although previous reports have described similar levels of agreement, very few of these studies have been carried out in children. Detjen et al [21] found an agreement of 95.6% (κ=0.91). Ferrara [14] reached a high agreement (κ=0.699), independently of the BCG vaccination status, but T-SPOT. *TB* detected a higher number of positive cases (38%) than QFN-G (26%). Furthermore, Kampmann et al [23] found a poorer agreement of IFN-γ tests (66.7%) in culture-confirmed TB cases, but the agreement was high (92%) in LTBI.
The analysis of discordant results needs to be researched further. This study has shown that when there is disagreement between both IFN-\(\gamma\) tests, a negative correlation exists between the number of SFCs and the amount of IFN-\(\gamma\) produced. In our study, in 11 cases the T-SPOT.\(TB\) was positive and the QFN-G-IT negative, and in 6 cases the T-SPOT.\(TB\) was negative but the QFN-G-IT positive. There may have been false positive or false negative IFN-\(\gamma\) tests. But it is also possible that there was an immunological dysfunction in these children. In fact, 3 of the children with a discordant result had discordant IFN-\(\gamma\) tests results again 3 months later. Recently, Richeldi et al [38] performed a comparative study on three different groups of immunocompromised individuals. They described highly discordant results, i.e., those clearly negative with one IFN-\(\gamma\) test and clearly positive with another, representing 12.1\% of the study population. These results suggest an immunological dysfunction related with a decreased production of IFN-\(\gamma\) or a decrease in the number of IFN-\(\gamma\) producing cells. Both situations have been associated with increased risk of developing active TB.

TB infection control in animals and humans is associated with the production of IFN-\(\gamma\) by the Th CD4+ cells [39]. It has been shown, in animal models, that a decreased production of IFN-\(\gamma\) and a decrease in the number of IFN-\(\gamma\) producing cells are predictive of an increased risk of developing TB [40]. In contact patients, it has been observed that the progression to disease was associated with a decrease in IFN-\(\gamma\) and an increase in IL-10 and IL-4 levels [41, 42]. Some data suggest that in individuals with a recent exposure to TB the protective response shifts from Th1 to Th2, even before the clinical symptoms appear [43]. Perhaps children with discordant IFN-\(\gamma\) tests could be a high risk group to developing TB, and therefore this could constitute a group that would benefit most from TB infection treatment.

In conclusion, in the daily practice of a referral centre for TB control, the use of IFN-\(\gamma\) tests is helpful for the diagnosis of TB infection. Its use eliminates the cross-reactions with BCG immunisation and may help to exclude NTM infections. The analysis of highly discordant results requires further investigation to elucidate any possible clinical implications. The use of both techniques simultaneously can contribute to improving the knowledge TB immunity.
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**Conflict of Interest.** None of the investigators have any financial interest in or a financial conflict with the subject matter or materials discussed in this manuscript. None of the Scientific Societies, nor Inverness Medical Ibérica SAU (Barcelona, Spain), Cellestis (Carnegie, Australia) or Oxford Immunotec (Abingdon, UK) had a role in the study design, implementation, data collection, management, analysis, interpretation of the data, preparation, review, or approval of the manuscript.
REFERENCES


gamma release assays improve the diagnosis of tuberculosis and nontuberculous
mycobacterial disease in children in a country with a low incidence of tuberculosis.
22. Connell TG, Curtis N, Ranganathan SC, Buttery JP. Performance of a whole blood
interferon gamma assay for detecting latent infection with Mycobacterium
Williams B, Crook AM, Hutton AM, Anderson ST. Interferon- 
gamma release assays do not identify more children with active TB than TST. Eur Respir J. 2009;
33: 1374-1382.
24. Dewan PK, Grinsdale J, Kawamura LM. Low sensitivity of a whole-blood interferon-
gamma release assay for detection of active tuberculosis. Clin Infect Dis. 2007; 44:
69-73.
25. Richeldi L. An update on the diagnosis of tuberculosis infection. Am J Respir Crit 
H, Wang H, Katsanis E. CD4(+)CD25(+)FoxP3(+) regulatory T cells suppress
27. Wilkinson RJ, Vordermeier HM, Wilkinson KA, Sjolund A, Moreno C, Pasvol G,
Ivanyi J. Peptide-specific T cell response to Mycobacterium tuberculosis: clinical
178: 760-768.
Altet N, Ausina V, Dominguez J. Quantitative evaluation of T-cell response after
specific antigen stimulation in active and latent tuberculosis infection in adults and
29. Haustein T, Ridout DA, Hartley JC, Thaker U, Shingadia D, Klein NJ, Novelli V,
Dixon GLJ. The likelihood of an indeterminate test result from a whole-blood
interferon-gamma release assay for the diagnosis of Mycobacterium tuberculosis
infection in children correlates with age and immune status. Pediatr Infect Dis J.
30. Lewinsohn DA. Embracing interferon-gamma release assays for diagnosis of latent
31. Haustein T, Ridout DA, Hartley JC, Thaker U, Shingadia D, Klein NJ, Novelli V,
Dixon GL. The likelihood of an indeterminate test result from a whole-blood


Table 1: Variables associated with a positive IFN-γ tests result: bivariate and multivariate analysis in the 166 children included in the study.

<table>
<thead>
<tr>
<th>Variable</th>
<th>T-SPOT. TB (n = 166)</th>
<th>QFN-G-IT² (n= 166)</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>Total</td>
<td>Positive (%)</td>
</tr>
<tr>
<td>Initial inclusion group</td>
<td></td>
<td></td>
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<td>Screening</td>
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<td>16 (23.5)</td>
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<td>Contacts</td>
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<td>48 (49.0)</td>
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<td>Yes</td>
<td>116 (69.9)</td>
<td>39 (33.6)</td>
</tr>
<tr>
<td>No</td>
<td>50 (30.1)</td>
<td>25 (50.0)</td>
</tr>
<tr>
<td>Chest X-ray :</td>
<td></td>
<td></td>
</tr>
<tr>
<td>Normal</td>
<td>152 (91.6)</td>
<td>53 (34.9)</td>
</tr>
<tr>
<td>Compatible with TB</td>
<td>14 (8.4)</td>
<td>11 (78.6)</td>
</tr>
</tbody>
</table>

¹Unadjusted Odds Ratio (95% confidence interval) for the positivity threshold of TST ≥5 mm; ²QuantiFERON-TB- Gold In Tube
Table 2: Concordance and agreement (Cohen’s Kappa coefficient) between the IFN-γ tests results for the different groups of children according to their BCG immunization status.

<table>
<thead>
<tr>
<th>IFN-γ results and agreements</th>
<th>Contact group</th>
<th>Initial inclusion group; n(%)</th>
<th>Screening group</th>
<th>Total (n=166)</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>BCG (n=68)</td>
<td>No BCG (n=30)</td>
<td>BCG (n=48)</td>
<td>No BCG (n=20)</td>
</tr>
<tr>
<td>Negative T-SPOT.TB and negative QFN-G-IT¹</td>
<td>36 (52.9)</td>
<td>7 (23.3)</td>
<td>35 (72.9)</td>
<td>15 (75.0)</td>
</tr>
<tr>
<td>Positive T-SPOT.TB and Positive QFN-G-IT</td>
<td>24 (35.3)</td>
<td>17 (56.7)</td>
<td>7 (14.6)</td>
<td>5 (25.0)</td>
</tr>
<tr>
<td>Negative T-SPOT.TB and positive QFN-G-IT</td>
<td>2 (2.9)</td>
<td>2 (6.7)</td>
<td>2 (4.2)</td>
<td>0</td>
</tr>
<tr>
<td>Positive T-SPOT.TB and negative QFN-G-IT</td>
<td>4 (5.9)</td>
<td>3 (10.0)</td>
<td>4 (8.3)</td>
<td>0</td>
</tr>
<tr>
<td>Failed T-SPOT.TB and positive QFN-G-IT</td>
<td>1 (1.5)</td>
<td>1 (3.3)</td>
<td>0</td>
<td>0</td>
</tr>
<tr>
<td>Failed T-SPOT.TB and negative QFN-G-IT</td>
<td>1 (1.5)</td>
<td>0</td>
<td>0</td>
<td>0</td>
</tr>
<tr>
<td>Overall Concordance² (%)</td>
<td>60/68 (88.2)</td>
<td>24/30 (80.0)</td>
<td>42/48 (87.5)</td>
<td>20/20 (100)</td>
</tr>
<tr>
<td>Cohen’s Kappa coefficient</td>
<td>0.765</td>
<td>0.561</td>
<td>0.622</td>
<td>1</td>
</tr>
</tbody>
</table>

Excluding failed results

| Concordance (%) | 60/66 (90.9) | 24/29 (82.8) | 42/42 (87.5) | 20/20 (100) | 146/163 (89.6) |
| Cohen’s Kappa coefficient | 0.810 | 0.609 | 0.622 | 1 | 0.778 |

¹QuantiFERON-TB Gold In Tube. ² Nº of patients with concordant results/total nº of patients.
Table 3: IFN-γ tests results according to the risk of exposure to *M. tuberculosis*.

<table>
<thead>
<tr>
<th>Risk Level</th>
<th>Number of Children</th>
<th>Number of Positive TST&lt;sup&gt;1&lt;/sup&gt; (%)</th>
<th>Positive IFN-γ tests&lt;sup&gt;2&lt;/sup&gt; (%)</th>
<th>Unadjusted OR (95% CI)&lt;sup&gt;3&lt;/sup&gt;</th>
<th>p value</th>
<th>Adjusted OR (95% CI)&lt;sup&gt;4&lt;/sup&gt;</th>
<th>p value</th>
</tr>
</thead>
<tbody>
<tr>
<td>No risk known</td>
<td>68</td>
<td>51 (75)</td>
<td>18 (35.3)</td>
<td>1</td>
<td>1</td>
<td></td>
<td></td>
</tr>
<tr>
<td>Medium risk</td>
<td>33</td>
<td>29 (87.9)</td>
<td>18 (62.1)</td>
<td>3.00 (1.06–8.64)</td>
<td>0.037</td>
<td>2.88 (1.22 – 6.80)</td>
<td>0.016</td>
</tr>
<tr>
<td>High risk</td>
<td>65</td>
<td>52 (80)</td>
<td>36 (69.2)</td>
<td>4.13 (1.68 – 10.27)</td>
<td>0.001</td>
<td>4.29 (2.01 – 9.18)</td>
<td>&lt;0.001</td>
</tr>
</tbody>
</table>

<sup>1</sup>Tuberculin skin testing results (≥ 5 mm in the contact group and TST ≥ 10 mm in the screening group was considered positive); <sup>2</sup>Positive result of one or both IFN-γ tests; <sup>3</sup>Unadjusted odds ratio (95% confidence interval); <sup>4</sup>Adjusted odds ratio (95% confidence interval) adjusted by age and gender.
Table 4: Number of spot forming cells after stimulation with ESAT-6, CFP-10 and RD1 antigens and the amount of IFN-γ released measured by T-SPOT.TB and QuantiFERON-Gold In Tube, respectively, in children diagnosed with active TB and LTBI infected (in both cases, either of the in vitro tests or both were positives).

<table>
<thead>
<tr>
<th>IFN-γ tests</th>
<th>Active TB</th>
<th>LTBI</th>
<th>p value</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>n</td>
<td>Median (25-75 percentiles)</td>
<td>n</td>
</tr>
<tr>
<td>QuantIFERON-Gold In Tube</td>
<td>9</td>
<td>2.09 (0.23-13.23)</td>
<td>52</td>
</tr>
<tr>
<td>T-SPOT.TB</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>ESAT-6</td>
<td>11</td>
<td>26.00 (5.00-69.00)</td>
<td>53</td>
</tr>
<tr>
<td>CFP-10</td>
<td>11</td>
<td>32.00 (18.00-75.00)</td>
<td>53</td>
</tr>
<tr>
<td>RD1</td>
<td>11</td>
<td>79.00 (37.00-137.00)</td>
<td>53</td>
</tr>
</tbody>
</table>
Table 5: Concordance and agreement (Cohen’s Kappa coefficient) between Tuberculin skin test and T-SPOT.TB and QuantiFERON-TB Gold In Tube results according to the BCG immunization status.

<table>
<thead>
<tr>
<th>BCG status</th>
<th>Tests compared</th>
<th>Cohen’s Kappa coefficient (SD)</th>
<th>p value</th>
</tr>
</thead>
<tbody>
<tr>
<td><strong>BCG immunized</strong></td>
<td>TST² and QFN-G-IT³</td>
<td>0.087 (0.155)</td>
<td>0.0048</td>
</tr>
<tr>
<td></td>
<td>TST and T-SPOT.TB</td>
<td>0.095 (0.151)</td>
<td>0.0032</td>
</tr>
<tr>
<td><strong>Not BCG immunized</strong></td>
<td>TST and QFN-G-IT</td>
<td>0.844 (0.105)</td>
<td>&lt;0.0001</td>
</tr>
<tr>
<td></td>
<td>TST and T-SPOT.TB</td>
<td>0.887 (0.062)</td>
<td>&lt;0.0001</td>
</tr>
<tr>
<td><strong>Total</strong></td>
<td>TST and QFN-G-IT</td>
<td>0.208 (0.111)</td>
<td>&lt;0.0001</td>
</tr>
<tr>
<td></td>
<td>TST and T-SPOT.TB</td>
<td>0.272 (0.092)</td>
<td>&lt;0.0001</td>
</tr>
</tbody>
</table>

¹SD: Standard Deviation; ²Tuberculin skin testing; ³QuantiFERON-TB Gold In Tube
FIGURE LEGENDS

Figure 1: IFN-γ tests results distribution (positive ■ and negative □) according to the tuberculin skin test induration in (A) BCG and (B) non-BCG-vaccinated children from contact and screening group.

Figure 2: IFN-γ tests results distribution (positive ■ and negative □) according to the tuberculin skin test induration in (A) contact and (B) screening group, including both BCG and non-BCG-vaccinated children.
Figure 3: Correlation between the number of spot forming cells (SFCs) after stimulation with specific *M. tuberculosis* antigens and the amount of IFN-γ released in children with concordant (A) and discordant (B) results between T.SPOT.TB and QFN-G-IT. The RD1 stimulation is the sum of SFCs obtained after ESAT-6 and CFP-10 stimulation. In children with concordant results the Pearson's correlation coefficient was 0.530 (p=0.0001). In children with discordant results the Pearson's correlation coefficient was -0.212 (p=0.371)