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Evaluation of quantitative interferon-γ responses for the follow up of children exposed to *Mycobacterium tuberculosis*

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ABSTRACT

Background and aims: Interferon-γ release assays (IGRAs) have been recently developed for the diagnosis of tuberculosis (TB) infection. The aim of the study is to evaluate the use of a commercial IGRA [QuantiFERON-TB Gold *In-Tube* (QFT-IT)] in the follow-up of children exposed to *Mycobacterium tuberculosis.*

Methods: A prospective study in 170 children at risk for TB infection was carried out. All children were tested with QFT-IT and tuberculin skin test (TST).

Results: One hundred and six children (62.4%) had taken therapy for active TB or latent tuberculosis infection (LTBI) and had undergone an end-of-therapy control with QFT-IT.

The QFT-IT became negative in 9/56 (16.1%) children. Neither the age (<o≥ 48 months) nor the diagnosis (active or LTBI) seem to be a risk factor for the results becoming negative.

Quantitative interferon-γ (IFN-γ) values did not decline significantly at the end of the therapy. On the other hand, a lower baseline IFN-γ level was more often associated with values of QFT-IT becoming negative at the end of the therapy (p=0.047). Moreover, the medians of the baseline IFN-γ values resulted significantly different between the children with active TB [6.75 (3.67–10.00) UI/ml] and those with LTBI [3.25 (1.00-7.98) UI/ml] (p=0.022).

Conclusions: At present, on the basis of our results as well as from published data, we believe that QFT-IT can have an important role for the follow-up of children at risk of TB infection. The quantitative response of QFT-IT rather than the qualitative results turns out to be important.

Key words: children, tuberculosis, interferon-γ, QuantiFERON-TB Gold *In-Tube*

INTRODUCTION

The World Health Organization estimates that one third of the world's population is infected with *Mycobacterium tuberculosis* and that, each year, about 9 million people develop tuberculosis (TB) of whom 2 million die [1]. One million of the annual TB cases occur in children [1]. Children are significantly more likely than adults to develop TB disease following primary *Mycobacterium tuberculosis* infection [2]. Children, especially those younger than 48 months, are also at much greater risk of developing severe clinical features, such as miliary disease and tuberculous meningitis [3]. Moreover a significant proportion of TB in adult arise from children with latent tuberculosis infection (LTBI) [2]. Treating infectious case as well as identifying children latently infected is therefore critical to control TB worldwide [4].

The diagnosis of TB infection in children is particularly challenging. To date, the gold standard for the diagnosis of TB disease is the detection of *Mycobacterium tuberculosis* [5]. However, smears from sputum or, more often, from gastric aspirates are positive in less than 15% of children diagnosed with TB and confirmation by culture is achieved only in less than 40% of cases [6,7]. Until recently the tuberculin skin test (TST) has been the only test available for the diagnosis of LTBI. The main drawback of the TST is its poor specificity in individuals sensitized by prior exposure to non-tuberculous mycobacteria (NTM) or by having been vaccinated with *Mycobacterium bovis* Bacille Calmette-Guérin (BCG) [8,9,10]. Additionally, TST sensitivity may be low in young children and in individuals with depressed immunity, malnutrition, or advanced TB [9,11].

New diagnostic blood tests, based on the detection of interferon-gamma (IFN-γ) released by specifically sensitized T cells, have been recently developed. They detect *in vitro* immune response to *Mycobacterium tuberculosis* by measuring, with enzyme-linked immunosorbent assay (ELISA) or with immunospot assay (ELISpot), the IFN-γ released from T cells in response to peptide antigens that simulate mycobacterial proteins [6,12,13]. The specific peptide antigens now available

are the early secreted antigenic target 6-kDa protein (ESAT-6), the culture filtrate protein 10-kDa (CFP-10) and the Rv2654 antigen (TB7.7). They are encoded by genomic segments of *Mycobacterium tuberculosis* which are absent from all BCG strains and from most NTM [6,12]. Two commercial ELISA-based IFN-γ release assays (IGRAs), Quanti-FERON-TB Gold (QFT-G, Cellestis) and the newer in-tube format (QFT-IT, Cellestis), have been both approved in Europe and by the United States Food and Drug Administration. A guideline from the Centers for Disease Control and Prevention (CDC) recommends the use of QFT-G in adults in all circumstances in which the TST is currently used [14]. The United Kingdom National Institute for Health and Clinical Exellence (NICE) TB guidelines recommend a two-step strategy for LTBI diagnosis with an initial TST, followed by an IFN-γ test in TST positive subjects or in those with less reliable TST testing [15].

Although IGRAs are considered very promising tests, they still leave unresolved issues such as the sensitivity and the specificity of these tests in the paediatric age. This issue is partially unresolved due to the absence of a gold standard for the LTBI and the frequent negative results in the diagnosis of TB disease. The trend in the IFN-γ production in response to specific mycobacterial antigens, both in TB disease and LTBI, may give useful indications regarding the diagnostic value. Particularly, the results becoming positive after exposure to a case of contagious TB disease, or the results becoming negative during the specific treatment, may represent indirect markers of the sensibility of these tests. An open question is whether they can be used for monitoring the efficacy of anti-tuberculosis treatment. A few studies published on adults and only two in children have provided contradictory results [16-20]. Nicol *et al*. have shown in ELISPOT an initial increase in the responses to purified protein derivative (PPD) after one month of therapy, followed by a decrease at 3 and 6 months. In contrast they have not found a significant difference in the response to ESAT 6 and CFP 10 between the beginning and the end of the treatment [19]. Herrmann *et al*. have not found a qualitative difference in the result of QFT at the end of the therapy. However they have shown an increase in the IFN-γ response at day 10 of treatment which might allow the

confirmation of a diagnosis. They have also found a decline in IFN-γ values during the treatment that, they state, make it possible for clinicians to monitor the effect of preventive or curative therapy [20].

The aim of this study was to determine how the qualitative and quantitative response of QFT-IT change during the follow-up throughout the treatment in children exposed to *Mycobacterial tuberculosis.*

MATERIALS AND METHODS

Study subject

Consecutive children (younger than 16 years) at risk for TB infection, who had referred to the Department of Paediatrics, University of Florence, Italy, were prospectively enrolled between June 2005 and June 2009. Patients were recruited from the Infectious Disease outpatient clinic (including a clinic dedicated to screening immigrants and internationally adopted children) and from inpatients admitted with symptoms suggestive of TB disease. The children eligible for the study were those belonging to the following three categories: a) children with clinical suspicion of TB disease; b) children in close contact with recently diagnosed cases of contagious TB disease; c) internationally adopted or recently immigrated children coming from countries with a high prevalence of TB. A maximum of two years was considered as period of recent immigration. Children with congenital or acquired immunodeficiency disorders (based on their medical history, clinical examination and/or laboratory tests) were excluded from the study.

Study design

Information regarding socio-demographic data, prior TB exposure, and past medical history was obtained from each child's parents or from medical documents. Children were considered to

have been vaccinated with BCG if there was a clear documentation and/or a BCG scar was present. In the absence of documentation or a BCG scar the vaccination status was considered unknown.

All children underwent clinical evaluation, venepuncture for QFT-IT at the beginning and at the end of the follow-up, and TST. Chest radiography was performed in all symptomatic children, in those with a positive TST, and in all contacts aged less than 5 years [21]. Children with suspected pulmonary TB had three sputa or early morning gastric aspirates samples collected for *Mycobacterium tuberculosis* detection (by means of microscopy, polymerase chain reaction and culture).

All results were recorded into the study database. The study was approved by local Ethics Committee and all parents of the enrolled patients gave written informed consent.

Methods

TST was administered according to the Mantoux method by injecting intradermally 5 tuberculin units (in 0.1 mL) of purified protein derivative (Biocine Test-PPD, Chiron, Siena, Italy) into the volar surface of the forearm. The TST result was read by experienced Infectious Disease specialists at 72 hours by measuring the transverse diameter of the skin induration at the site of the PPD application with a flexible ruler. Following the American Academy of Pediatrics guidelines [22], a positive TST was defined as an induration size ≥ 5 mm for children in close contact with known or suspected contagious case of TB disease or for children suspected to have TB disease (based on clinical evidence and/or chest radiograph) and ≥ 10 mm for children born in countries with a high prevalence of TB and recently immigrated.

TB disease diagnosis was assigned to any child with *Mycobacterium tuberculosis* cultured or detected by microscopy or molecular methods from sputum or gastric aspirate. For culture, both solid and liquid media (Löwenstein-Jensen and BACTEC 460TB, BD Biosciences Division, Sparks, Md;) were used. For children it is often difficult to obtain a positive result from the above investigations [6,7]. As a consequence, the TB disease diagnosis was also assigned to any child

with clinical and radiologic evidence of active TB, and with either a history of exposure to an infectious case or a positive TST [6,23]. In the absence of a recognized standard, LTBI diagnosis was assigned to any child with a positive TST and no clinical, bacteriologic or radiographic evidence of active TB [11]. All asymptomatic children with a negative TST were defined as uninfected.

QuantiFERON-TB Gold *In-Tube* (QFT-IT, manufactured by Cellestis Limited, Carnegie, Victoria, Australia) was performed in the immunology laboratory according to the manufacturer's instructions [24]. Laboratory personnel were blinded to the status of the patient and TST result. QFT-IT was supplied with three heparinized blood collection tubes precoated with three TB-specific antigens [ESAT 6, CFP-10 and TB7.7(p4)], a mitogen (phytohemagglutinin), and a negative control. The cut-off for a positive test, indicating likely *Mycobacterium tuberculosis* infection, was 0.35 IU/mL of IFN-γ for the TB specific antigen-stimulated plasma sample above the amount of IFN-γ in the negative control sample. A positive response to the TB-specific antigens without a response to the mitogen was considered valid and positive result. If there was no detectable IFN-γ response to the mitogen and the TB specific antigens or if the IFN-γ level in the negative control was too high, the test was deemed "indeterminate". Starting from January 2006, quantitative data of IFN-γ response to TB specific antigen and to mitogen were also recorded.

Statistical analysis

Age, TST size and IFN-γ values were expressed as median and interquartile range (IQR) of months, millimeters and IU/mL, respectively. The kappa (*k*) statistic was used to assess the agreement between two tests. Differences in frequencies were evaluated by the Fisher test and the X^2 test. Mann Whitney test and Kruskal-Wallis test were used to compare the medians. Differences between the IFN-γ at the beginning and end of therapy were evaluated by Wilcoxon test for paired data. Data were analyzed using SPSS 11.5 (Statistical Package for Social Science, Chicago, III). Significance was defined by $p \leq 0.05$.

RESULTS

One hundred-seventy children were enrolled in the study. The median age was 76.0 (39.0 - 109.0) months. Their demographic characteristics and the reasons for testing are summarized in Table 1.

The diagnosis of active TB disease was made in 21 (12.3%) children. All these children presented with pulmonary disease. One-hundred and three children (60.6%) were diagnosed with LTBI and 46 (27.1%) children were defined as uninfected. Table 2 shows the BCG status, the TST size, and the QFT-IT results according to diagnosis.

QFT-IT was positive in 66 (38.8%) children and negative in 101 (59.4%). Three (1.8%) children had an indeterminate result. In 115 (68.9%) out of 167 cases there was concurrence of the TST and QFT-IT results. The overall agreement between the QFT-IT and the TST (without considering the indeterminate results) was low with a *k* value of 0.361. The median age of children with a positive QFT-IT was 97.5 (70.5-123.5) months whereas the median age of those with a negative result was 50 (830-99) months. At the time of the diagnosis, the QFT-IT was positive in 17 of 21 (81.0%) children diagnosed with TB disease. Of the 103 children defined as LTBI according to the TST results, 47 (45.6%) had a positive and 54 (52.4%) had a negative QFT-IT result; 2 (1.9%) children had an indeterminate result. Of the 46 remaining children, defined as uninfected, 43 (93.5%) were tested negative, 2 (4.3%) were positive and 1 (2.2%) indeterminate.

The median of the IFN-γ values at the time of the diagnosis in children with LTBI (3.25 [1.00-7.98] UI/ml) was significantly lower than that of those with TB disease (6.75 [3.67-10.0] UI/ml), (p =0.022).

Follow-up. One hundred and six (62.4%) out of 170 children enrolled into the study had taken therapy for active TB or LTBI and had undergone an end-of-therapy control with QFT-IT. Four of them had taken the preventive therapy for LTBI, although they had been defined as uninfected, because they were very close contact of contagious TB cases. Twenty (11.8%) children,

who had been defined as uninfected and had a contact with contagious TB disease, had taken a therapy only in the "window period" between the first and the second TST. Twenty-four (14.1%) children had a QFT follow-up, but had not taken any therapy. Among these, 2 children with LTBI were not treated because one had had an adverse reaction to both Isoniazide (INH) and Rifampicin (RMP) and the other was a close contact of an index case who was resistant to all anti-tuberculosis therapy. Twenty children had taken therapy for LTBI or active TB, but the end of therapy control with QFT-IT was not performed (8 children are still in follow up and 12 children were lost to follow up).

We analyzed, in particular, whether there were qualitative and quantitative changes of QFT-IT at the beginning and end of therapy. Figure 1 shows the qualitative results. In 91 (85.8%) out of 106 children treated with specific therapy, the QFT-IT result did not change at the end of the therapy. The QFT-IT became negative in nine (16.1%) out of 56 children with a positive QFT-IT at the beginning of the therapy and became positive in 4 (8.3%) out of 48 children with previous negative result.

Children with a positive result at the time of the diagnosis were further analyzed in order to understand what factors may have an influence on the result becoming negative. In 47 (83.9%) out of 56 children with a positive result at the diagnosis, the QFT-IT result remained positive at the end of the therapy. In these children the median of the IFN-γ values at the beginning and at the end of the therapy was 5.64 and 4.55 UI/ml respectively. This difference was not statistically significant (p=0.474) although a decreasing trend was appreciable.

One (11.1%) out of 9 children whose result had become negative at the end of the therapy was younger than 48 months and 8 (88.9%) children were older than 48 months. Among the children whose result had remained positive at the end of the therapy, 5 (10.6%) out of 47 were younger than 48 months and 42 (89.4%) were older than 48 months. There was no significant difference between the two groups in being younger or older than 48 months (p=1.000).

Considering the diagnosis, 8 (88.9%) out of 9 children whose result had become negative had a diagnosis of LTBI and 1 (11.1%) of active TB. Among the children whose result had remained positive at the end of the therapy, 34 (72.3%) out of 47 were diagnosed with LTBI and 13 (27.7%) with active TB. There was no significant difference between the two groups in having a diagnosis of LTBI or active TB. (p=0.424).

Finally, quantitative IFN-γ values before the treatment were analyzed. The baseline IFN-γ value in children whose result had become negative was significantly lower than the IFN-γ value in children whose result had remained positive (1.08 [0.66-4.98] UI/ml *vs.* 5.64 [1.99-9.82] UI/ml [p=0.047])

It was then investigated whether the different therapy may have affected the test result. Children with LTBI were treated with INH for 6-9 months (23 children) or with INH and RMP for 3 months (62 children). In case of contact with contagious TB disease resistant to any of these drugs, a scheme with INH, RMP and Pyrazinamide (PZA) for the first 2 months (4 children) was used. Children with TB disease were treated with INH and RMP (for 6-10 months) + PZA (for 2 months) + Ethambutol (EMB) (for 4 months) (4 children).

Among the children with a positive result before the treatment, the QFT-IT result became negative in 3 (3%) out of 8 children treated with INH, in 4 (12.9%) out of 31 children treated with INH+RMP, in 1 (16.7%) out of 6 children treated with INH+RMP+PZA and in 1 (9.1%) out of 11 children treated with INH+RMP+PZA+EMB.

Table 3 shows the median of the IFN-γ values at the beginning and end of the treatment according to the different therapeutic scheme. The IFN-γ values tended to decrease at the end of the therapy, although the difference was not statistically significant in any of the therapeutic schemes.

The follow up of the 24 children (2 with LTBI and 22 defined as uninfected) that were not treated was also evaluated. The QFT-IT results in the 2 children with LTBI (one negative and one positive) did not change at the end of the therapy. Among the 22 children uninfected, 2 (9%)

children had a positive result at the baseline that became spontaneously negative at the follow up. The remained 20 children were negative at the baseline and at the end of the follow up.

DISCUSSION

This work represents, to date, the pediatric prospective study with the largest number of cases evaluating the trend of the IFN-γ production in response to specific mycobacterial antigens in the follow-up of children exposed to *Mycobacterium tubercolosis*. Our results show that the QFT-IT becomes negative at the end of the anti-tuberculosis therapy in approximately 16% of the children with TB infection and with a positive QFT-IT at the baseline. At the end of the therapy a decrease of the quantitative IFN-γ values was also noted, although this was statistically not significative. Among the results of this study, particularly relevant is the demonstration that a lower baseline IFN-γ value was more often associated with values of QFT-IT becoming negative at the end of the therapy. Moreover, the baseline IFN-γ value in children with LTBI resulted significantly lower than the IFN-γ value in children with TB disease. Finally, the age (being older or younger than 48 months), the diagnosis (TB disease or LTBI) and the therapeutic scheme do not seem to be a risk factor for the results becoming negative. On the other hand, in a few children diagnosed with LTBI a negative QFT-IT at the baseline became positive at the end of the therapy. Probably these children had been recently exposed to the infection of *Mycobacterium tuberculosis*. The discrepancy between TST (positive) and QFT-IT (negative) results at the diagnosis, with QFT-IT later becoming positive, could be associated to two main reasons: firstly, to a booster effect resulting from the infection of *Mycobacterium tuberculosis* in individuals sensitized by prior exposure to mycobacterial antigens (as a consequence of the vaccination with BCG or infection from NTM), secondly, to TST effectively becoming positive sooner than QFT-IT. This evidence is not confirmed by other data from the literature. If we had followed the NICE guidelines, which indicate

that only children with both tests (TST and IGRA) positive should be treated, then these children would not have been treated.

In agreement with our results, Herrmann *et al*., within a pediatric field, and Sauzullo *et al*. in adults, have demonstrated a decrease of IFN-γ at the end of the therapy which, in their case, results statistically significant [20,17].

The main difficulties that we met in carrying out our study were due to the high number of adopted and immigrated children, since in these cases the information regarding medical history and social data were often missing. A limiting factor in our study was that we could investigate a relatively small number of children with a diagnosis of active TB. Furthermore, not all the children had a control test of QFT-IT and a longer follow-up is missing, specially after the end of the therapy. Moreover, it was not available a control group of children with LTBI who were not treated, due to ethical reasons.

At present, on the basis of our results as well as from published data, we believe that QFT-IT can have an important role for the follow-up of children at risk of TB infection. In particular, the quantitative response of QFT-IT rather than the qualitative results turns out to be important. Undoubtedly, long-term studies are necessary in order to confirm the validity of QFT-IT, which has, anyhow, the potential to become a useful diagnostic tool for TB control.

Table 1: Socio-demographic and clinical characteristics of study population

	total
	n (%)
< 48 months	59 (34.7)
male	93 (54.7)
region of origin	
Italy	27 (15.9)
Sub-Saharian Africa	9 (5.3)
North-Africa	8 (4.1)
Asia	32 (18.8)
Eastern-Europe	51 (30.0)
Latin-America	43 (25.3)
reason for testing:	
screening	
recently immigrated	12 (7.1)
internationally adopted	67 (39.4)
contact	
immigrants	49 (28.8)
Italians	22 (12.9)
adopted	2 (1.2)
clinical suspicion of active pulmonary TB	
immigrants	11 (6.5)
adopted	1 (0.6)
Italians	6 (3.5)

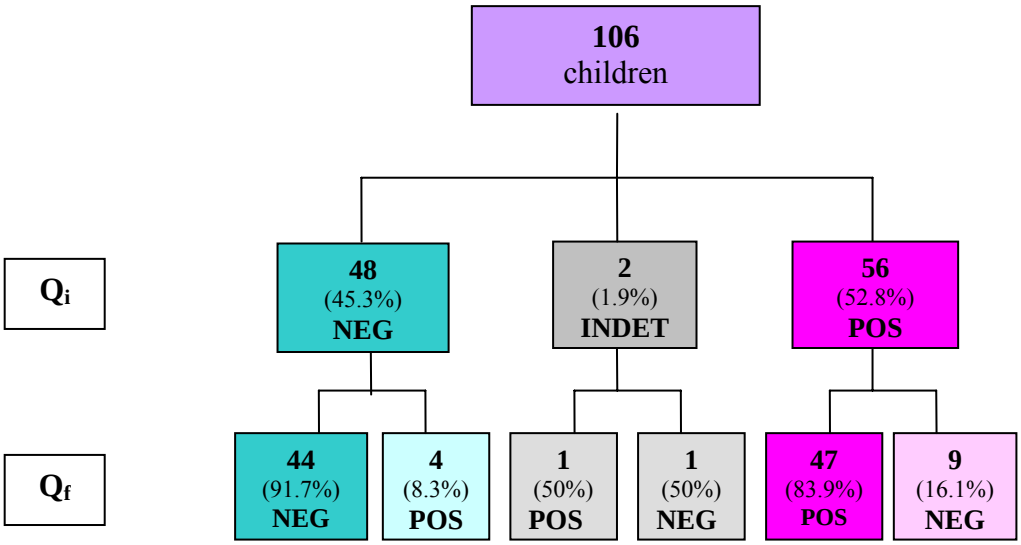
TB = tuberculosis

Table 2: BCG vaccination status, TST size and QuantiFERON-TB Gold *In-Tube* result according to diagnosis

	uninfected	**latent TB**	**TB disease**	**total**
	n = 46	**n = 103**	**n = 21**	**n = 170**
	n (%)	n (%)	n (%)	n (%)
BCG vaccination				
yes	3 (6.5)	65 (63.1)	5 (23.8)	73 (42.9)
no	36 (78.3)	16 (15.5)	10 (47.6)	62 (36.5)
unknown	7 (15.2)	22 (21.4)	6 (28.6)	35 (20.6)
TST induration diameter (mm)				
< 5	46 (100)	2 (1.9)	2 (9.5)	50 (29.4)
≥ 5 and < 10	0	15 (14.6)	0	15 (8.8)
≥ 10 and < 15	0	28 (27.2)	9 (42.9)	37 (21.8)
≥ 15	0	58 (56.3)	10 (47.6)	68 (40.0)
QFT-IT result				
negative	43 (93.5)	54 (52.4)	4 (19.0)	101 (59.4)
positive	2 (4.3)	47 (45.6)	17 (81.0)	66 (38.8)
indeterminate	1 (2.2)	2 (1.9)	0	3 (1.8)

BCG = Bacille Calmette-Guérin, TST = tuberculin skin test, QFT-IT = QuantiFERON-TB Gold *In-Tube*

Figure 1. QuantiFERON-TB Gold *In-Tube* (QFT-IT) results at the beginning and at the end of the anti-tuberculosis therapy.



Q_i = QFT-IT initial, Q_f = QFT-IT final

Table 3. Median and *Range* Interquartile (IQR) of interferon-γ. (UI/ml) values at the time of the diagnosis and at the end of the anti-tuberculosis therapy, according to the different therapeutic schemes.

	IFN-γ (UI/ml) BEFORE THERAPY Median (IQR)	**IFN-γ (UI/ml) END THERAPY Median (IQR)**	**p**
INH	8.67 (5.62 -10.82)	5.15 (2.92-7.34)	n.s. (0.500)
INH+RMP	4.12 (1.9-8.3)	4.33 (1.5-8.9)	n.s. (0.086)
INH+RMP+PZA	4.67 (3.96-7.29)	3.34 (2.7-14.76)	n.s (0.869)
INH+RMP+PZA+EMB	6.21 (3.67-15.5)	5.32 (2.51-6.8)	n.s. (0.684)

INH=Isoniazide, RMP=Rifampicin, PZA=Pyrazinamide, EMB=Ethambutol

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