Journal of Infection (2014) xx, 1–10

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IP-10 is an accurate biomarker for the diagnosis of tuberculosis in children

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Accepted 3 June 2014
Available online ■ ■ ■

KEYWORDS
IP-10;
IFN-γ;
Tuberculosis;
Children

Summary *Objective:* Performance of IFN-γ assays in children is compromised. Therefore, we investigated the utility of IP-10 for the detection of active tuberculosis (TB) and latent tuberculosis infection (LTBI) diagnosis in children; comparing its positivity with QuantiFERON-TB Gold In-Tube (QFN-G-IT) and T-SPOT.TB.
Methods: We studied 230 children from three groups: active TB, screening (healthy children without known exposure to active TB patient screened at school or by their paediatrician) and contact-tracing studies. IFN-γ release was determined by QFN-G-IT and T-SPOT.TB. IP-10 was detected in QFN-G-IT supernatants by ELISA.
Results: When combining QFN-G-IT and IP-10 assays, positive results improved significantly from 38.3% in QFN-G-IT and 33.9% in IP-10 to 41.3%. Age and type of contact were significant risk factors associated with positive QFN-G-IT and IP-10 results. IP-10 levels after antigen-specific

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http://dx.doi.org/10.1016/j.jinf.2014.06.013

stimulation were significantly higher in comparison to IFN-γ levels. Correlation between the three assays was good ($\kappa = 0.717-0.783$).

Conclusions: IP-10 cytokine is expressed in response to TB specific-antigens used in QFN-G-IT. In conclusion, the use of IFN-γ T-cell based assays in combination with an additional IP-10 assay detection could be useful for diagnosing active TB and LTBI in children.

Background

In 2011, there were an estimated 8.7 million incident cases of tuberculosis (TB) of which approximately 0.5 million cases and 64.000 deaths occurred in children.[1,2] TB in children is difficult to diagnose due to paucibacillary disease and uncharacteristic clinical presentation; as a consequence, TB epidemic in this population is underestimated. So, there is a need to improve diagnostic tools for TB disease and infection in children. One approach is the identification of new specific biomarkers that potentially allow selecting children that are at higher risk of developing active TB.

Immunodiagnostic tests such as the tuberculin skin test (TST) and Interferon-γ release assays (IGRAs) are the only available tools for diagnosing LTBI. They are also used as complementary assays for diagnosing active TB in children. The TST has poor specificity due to its cross-reaction with *Mycobacterium bovis bacillus Calmette-Guérin* (BCG) vaccine strain and non-tuberculous mycobacteria (NTM) and poor sensitivity in high-risk individuals with an impaired cellular immunity such as children under the age of 5.

IGRAs, introduced a decade ago, rely on the detection of IFN-γ secreted by effector T cells after the stimulation with specific *Mycobacterium tuberculosis* (MTB) antigens (ESAT-6, CFP-10 and TB7.7).[3] Currently, there are two U.S. Food and Drug Administration approved IFN-γ T-cell based assays: QuantiFERON-TB Gold In-Tube (QFN-G-IT, QIAGEN, Düsseldorf, Germany) and T-SPOT.TB (Oxford Immunotec, Abingdon, UK). QFN-G-IT test stimulates whole-blood with ESAT-6, CFP-10 and TB7.7 in the same tube, and measures the concentration of IFN-γ in supernatants with an enzyme-linked immunosorbent assay. T-SPOT.TB assay stimulates isolated peripheral blood mononuclear cells with ESAT-6 and CFP-10 separately, and detects the number of IFN-γ producing T cells by means of an enzyme-linked immunospot assay (ELISPOT). Both assays are useful techniques for LTBI diagnosis because they avoid false-positive results in BCG-vaccinated individuals and most of NTM.[4–7] In addition, they can help in the immunodiagnosis of active TB.[8] On the contrary, although IFN-γ based assays are more sensitive than TST in immunosuppressed patients, their sensitivity is still compromised.[9] Furthermore, reported data about performance of IFN-γ assays in children, especially in those younger than five years old, is still questionable.[10,11]

IFN-γ inducible protein 10 (IP-10) is a chemokine mainly expressed by antigen-presenting cells, and induced by innate and adaptive mechanisms. During adaptive immune response, its secretion is mainly driven by T-cell-derived IFN-γ.[11–13] It has been reported that IP-10 levels are higher than those of IFN-γ, suggesting that it can be a promising diagnostic marker in some infectious diseases as TB and hepatitis C virus (HCV).[13–17] In the context of TB diagnosis, IP-10 response is known to be more robust than IFN-γ in HIV-infected patients and young children.[18–22] However, although there are some studies available, comparison of both IFN-γ assays with IP-10 biomarker is still limited in children and should be further investigated.

In this study, we explored the utility of the IP-10 cytokine detection by an *in-house* ELISA for active TB and LTBI diagnosis in children; and we compared its positivity with QFN-G-IT and T-SPOT.TB.

Materials and methods

Patients and inclusion criteria

We retrospectively enrolled a total of 230 paediatric patients, who attended to the TB Control and Prevention Unit of Barcelona (CAP Drassanes, Barcelona, Spain), Hospital Clínico Universitario de Valencia (Valencia, Spain) or Hospital Universitari Germans Trias i Pujol (Badalona, Spain) with suspected active TB or LTBI. Ethics approval for this study was provided by the corresponding Ethics Committees. We obtained written informed consent from all parents before the blood sampling. A detailed questionnaire from all paediatric patients was collected in order to indicate: gender, age, place of birth, immigration status, results of any previous TST, BCG-vaccination status, details of any contact with an active TB patient, history of previous active TB, chest radiograph and other medical conditions like immunosuppression status. In this study, only participants with a BCG scar were considered for analysis as BCG vaccinated.

Patients included in the study were classified in three groups. Group 1: 12 children diagnosed with active TB. Inclusion criteria were a positive culture for MTB; group 2: 81 children tested using IGRA during contact-tracing after exposure to an active TB patient; group 3: 137 healthy children, without known exposure to an active TB case, examined during LTBI screenings because of routine examinations at school or by their paediatrician. The main demographic characteristics of patients included in the study are summarized in Table 1.

Quanti-*FERON* TB Gold *In Tube*

QFN-G-IT was performed according to the manufacturer's instructions. Results were considered positive if the antigen-specific and mitogen (positive control) values were equal or higher than 0.35 IU/mL or 0.5 IU/mL of IFN-γ, respectively. The test was considered indeterminate

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Table 1 Patient's demographic characteristics.

Variable	Active TB $n = 12$ (%)	Contact-tracing studies $n = 81$ (%)	LTBI screening $n = 137$ (%)
Gender			
Male	7 (58.3)	36 (44.4)	82 (59.9)
Female	5 (41.7)	45 (55.6)	55 (40.1)
Age, mean ± SD	10.50 ± 6.42	9.93 ± 3.87	9.93 ± 4.32
BCG-vaccinated			
Yes	5 (41.7)	50 (61.7)	76 (55.5)
No	7 (58.3)	31 (38.3)	61 (44.5)
Birth country			
Immigrants from countries with high prevalence of TB infection	6 (50)	52 (64.2)	74 (54)
Residents in a non-epidemic TB country	6 (50)	29 (35.8)	63 (46)

if the response to the specific MTB antigens was negative and if the response to the mitogen was less than 0.5 IU/mL and/or the value of the negative control was higher than 8.0 IU/mL. All the results from the nil control were subtracted from the mitogen and the antigen-specific values.

T-SPOT.TB

T-SPOT.TB was also performed following the manufacturer's recommendations. Tested wells were scored as positive if they contained at least six spot forming cells (SFC) more than in the negative control well and if this number was at least twice the number of the negative control well. A result was considered indeterminate if the antigen-stimulated sample was negative and if the value of the positive control was less than 20 SFC or/and the number of SFC in the negative control was greater than 10. All SFCs of the nil control were subtracted from the SFCs of mitogen and the antigen-specific wells. SFCs were analysed with an automated AID ELISPOT plate reader (Lector AID ELISPOT, Autoimmun Diagnostiks GMBH, Germany) and simultaneously naked eye verified.

IP-10 *in-house* ELISA

IP-10 was retrospectively detected in QFN-G-IT supernatants by a non-commercial *in-house* ELISA. All ELISA reagents were prepared and developed in Copenhagen (Denmark), and the tests were performed in Badalona (Spain). Plasma samples were diluted 33 times and IP-10 concentration was measured as described previously.[23] IP-10 cut offs were set up using ROC curve analysis (at highest sensitivity with minimal loss of specificity) as previously described and established.[24] Positive and indeterminate cut offs for IP-10 test were 2.4 ng/mL and 1.5 ng/mL.[24] For establishing an IP-10 specificity value, we checked IP-10 responses in 21 TST negative healthy children coming from routine LTBI screenings at school (Group 3, LTBI screening).

Statistical methods

Comparison of IFN-γ/IP-10 concentrations among the three study groups was performed using the Kruskall–Wallis test. Mann–Whitney U analysis was applied for pairwise comparisons with a Bonferroni-Dunn *post hoc* correction. Differences were considered statistical significant when a *p*-value was <0.05. However, when 3 groups were compared, a *p*-value <0.016 was considered significant after the Bonferroni-Dunn correction. Risk factors for a positive test result were defined using odds ratio (OR). All variables included in the multivariate analysis were determined a priori based on an estimation of their significance during univariate analysis. Test concordance was assessed using Cohen's Kappa (κ) coefficient. *K* values below 0.40 indicate weak agreement, values between 0.41 and 0.60 indicate good agreement and values above 0.60 indicate strong agreement. Association between IFN-γ/IP-10 concentrations after mitogen stimulation with age was assessed using Spearman correlation coefficient. All analyses were made with SPSS statistical software for Windows (SPSS version 15.0; SPSS, Chicago, IL). Graphical representation is based on GraphPad Prism version 4 (GraphPad Software, San Diego, CA).

Results

QFN-G-IT and IP-10 ELISA in-house tests performance

The overall number of positive results, considering all children population was 38.3% and 33.9% using QFN-G-IT and IP-10 assay, respectively. There were significant differences in the percentages of positive results between the two tests ($p = 0.034$). When QFN-G-IT and IP-10 assays were combined, overall positive results improved significantly to 41.3% ($p < 0.005$) (Table 2). We obtained one indeterminate result (0.4%) by QFN-G-IT and two (0.87%) by IP-10 assay. For all cases this was due to an insufficient response to phytohaemagglutinin (PHA) and MTB specific antigens. One of the two indeterminate results by IP-10 tests was very close to the mitogen-stimulated threshold.

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Table 2 Distribution of QFN-G-IT and IP-10 results in the different study groups.

		QFN-G-IT	IP-10 test	QFN-G-IT + IP-10
Overall	Positive	88 (38.3)	78 (33.9)	95 (41.3)*
	Negative	141 (61.3)	150 (65.2)	135 (58.7)
	Indeterminate	1 (0.4)	2 (0.9)	0 (0)
Active TB	Positive	11 (91.7)	8 (66.7)	11 (91.7)
(n = 12)	Negative	1 (8.3)	4 (33.3)	1 (8.3)
	Indeterminate	0 (0)	0 (0)	0 (0)
Contact-tracing studies	Positive	37 (45.7)	38 (46.9)	41 (50.6)τ
(n = 81)	Negative	44 (54.3)	43 (53.1)	40 (49.4)
	Indeterminate	0 (0)	0 (0)	0 (0)
LTBI screening	Positive	40 (29.2)	32 (23.3)	43 (31.4)ŧ
(n = 137)	Negative	96 (70.1)	103 (75.2)	94 (68.6)
	Indeterminate	1 (0.7)	2 (1.5)	0 (0)

* $p < 0.005$ compared to QFN-G-IT and IP-10.
τ $p = 0.046$ compared to QFN-G-IT.
ŧ $p = 0.046$ and $p < 0.0001$ compared to QFN-G-IT and IP-10, respectively.

The distribution of positive, negative and indeterminate QFN-G-IT and IP-10 results in the different study groups is shown in Table 2. Considering active TB children, sensitivities of IFN-γ and IP-10 assays were 91.7% and 66.7%, respectively. When combining QFN-G-IT and IP-10 assay the sensitivity did not improve in comparison to QFN-G-IT alone (91.7%). When both assays were pooled for the other two study groups, the percentage of positive results increased significantly respect QFN-G-IT to 50.6% in children enrolled during contact-tracing studies ($p = 0.046$); and increased significantly with respect to QFN-G-IT and IP-10 assay to 31.4% in children screened for LTBI ($p = 0.046$ and $p < 0.0001$ respectively). IP-10 responses were negative in all 21 TST negative healthy children coming from LTBI screening group. In addition, IFN-γ responses were also negative.

Risk factors associated with a positive QFN-G-IT and IP-10 assay are shown in Tables 3 and 4. On univariate analysis, age and type of contact were statistically significant for positive QFN-G-IT and IP-10 results. In the multivariate analysis, age, birth country and type of contact showed a significant association for a positive QFN-G-IT. However, for IP-10 analysis only age and type of contact were found statistical significant.

We compared the overall amount of cytokines released after specific antigen stimulation between different groups of all children enrolled in the study (Fig. 1). A significant difference in IFN-γ/IP-10 released after MTB-specific

Table 3 Association between tuberculosis risk factors and positive QFN-G-IT, IP-10 and T-SPOT.TB results by means of univariate analysis.

Risk factors	QFN-G-IT[a]		IP-10[a]		T-SPOT.TB[a]	
	Positive (n)	P	Positive (n)	P	Positive (n)	P
Gender						
Men	44 (37.6)	NS	36 (30.8)	NS	30 (33.3)	NS
Women	33 (33)		34 (34.3)		26 (33.8)	
Age years	—	0.002	—	0.002	—	NS
Birth country						
Low prevalence	38 (41.3)	NS	32 (34.8)	NS	25 (44.6)	0.031
High prevalence	39 (31.2)		38 (30.6)		31 (27.9)	
Type of contact						
Screening	40 (29.4)	0.001	32 (23.7)	0.000	20 (22)	0.001
>6 h	21 (47.7)		21 (47.7)		21 (51.2)	
<6 h	2 (13.3)		3 (20)		4 (26.7)	
Sporadic	14 (63.6)		14 (63.6)		11 (55)	
Index case[b]						
Smear-positive	20 (51.3)	NS	20 (51.3)	NS	19 (52.8)	NS
Smear-negative	5 (33.3)		4 (26.7)		5 (33.3)	

NS: Non significant differences.
[a] Patients with an indeterminate QFN-G-IT, IP-10 or T-SPOT.TB response were excluded from the analysis.
[b] LTBI screenings were excluded.

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Table 4 Association between tuberculosis risk factors and positive QFN-G-IT, IP-10 and T-SPOT.TB results by means of multivariate analysis.

Risk factors	QFN-G-IT[a]		IP-10[a]		T-SPOT.TB[a]	
	OR (CI95%) adjusted	*P*	OR (CI 95%) adjusted	*P*	OR (CI 95%) adjusted	*P*
Gender						
Men	1		1		1	
Women	0.731 (0.395–1.353)	NS	1.028 (0.548–1.926)	NS	0.793 (0.391–1.608)	NS
Age years	1.140 (1.056–1.231)	0.001	1.141 (1.055–1.235)	0.001	1.070 (0.981–1.167)	NS
Birth country						
Low prevalence	1		1		1	
High prevalence	0.500 (0.266–0.937)	0.031	0.637 (0.335–1.211)	NS	0.477 (0.231–0.985)	0.045
Type of contact						
Screening	1		1		1	
>6 h	2.847 (1.344–6.030)	0.006	3.501 (1.635–7.500)	0.001	3.995 (1.757–9.083)	0.001
<6 h	0.455 (0.092–2.244)	NS	0.930 (0.231–3.745)	NS	1.515 (0.417–5.507)	NS
Sporadic	4.131 (1.561–10.930)	0.004	5.401 (2.040–14.305)	0.001	3.940 (1.391–11.161)	0.01

NS: Non significant differences.
[a] Patients with an indeterminate QFN-G-IT, IP-10 or T-SPOT.TB response were excluded from the analysis.

stimulation was found within the evaluated groups (Kruskall–Wallis test: $p = 0.0004$ for IFN-γ and $p = 0.0001$ for IP-10). Furthermore, pairwise comparisons showed that IFN-γ and IP-10 released in active TB patients was significantly higher than in children screened for LTBI (Fig. 1A and B). When we analysed the secreted amount of both cytokines only in those children with a positive response for QFN-G-IT and IP-10 assay, differences between children with active TB respect the other LTBI groups were not significant (Fig. 1C and D).

Levels of IP-10 released after antigen-specific stimulation were compared with levels of IFN-γ released detected by QFN-G-IT. For the three groups considered, we observed significant higher levels of IP-10 in relation to IFN-γ (Fig. 2).

We also analysed the possible impact of age, studying T cell responses on QFN-G-IT and IP-10 assay. As a result, no correlation with age was observed when analysing the concentration of IFN-γ and IP-10 released after mitogen stimulation (Spearman's rho [SR] = -0.009, $p = 0.933$ and SR = 0.108, $p = 0.296$, respectively; Figure not shown).

QFN-G-IT, IP-10 and T-SPOT.TB results

T-SPOT.TB results were available in a subgroup of 181/230 (78.7%) patients. Overall number of positive results was 37.6%, 34.8% and 37% for QFN-G-IT, IP-10 assay and T-SPOT.TB respectively (Table 5). There were no significant differences in the percentages of positive results between the three tests ($p = 0.358$). In this subgroup of patients, we found one indeterminate result (0.6%) with QFN-G-IT, two with IP-10 assay (1.1%) and three with T-SPOT.TB (1.7%). One T-SPOT.TB indeterminate result was due to an excessive response of specific-sensitized T cells in the negative control.

The distribution of positive, negative and indeterminate QFN-G-IT, IP-10 and T-SPOT.TB results in the different study groups is shown in Table 5. Considering the active TB group, sensitivity of both QFN-G-IT and T-SPOT.TB assays was 91.7%. In contrast, sensitivity of IP-10 assay was 66.7%. Combining IP-10 and T-SPOT.TB, the sensitivity increased to 100%, being this difference statistically significant for IP-10 assay ($p = 0.046$). However, when combining QFN-G-IT and IP-10 assay, in this subgroup, the sensitivity did not improved in comparison to QFN-G-IT alone (91.7%).

When we analysed factors associated with positive T-SPOT.TB result, birth country and type of contact were statistically significant in univariate and multivariate analysis (Tables 3 and 4).

Concordance and agreement between QFN-G-IT, IP-10 and T-SPOT.TB

The agreement and concordance between the three assays for the different groups of children are shown in Table 6. Global agreement between QFN-G-IT and IP-10 assay was 89.4% ($\kappa = 0.773$; standard error [SE] = 0.044). The overall agreement, for the subgroup of patients with results for the 3 tests, agreement between IP-10 and QFN-G-IT with T-SPOT.TB was 86.9% ($\kappa = 0.717$; SE = 0.055) and 89.8% ($\kappa = 0.783$; SE = 0.048), respectively. When κ values were calculated for each group of patients, we found that QFN-G-IT and IP-10 assays showed a weak correlation in the group of children with active TB and a strong correlation for the rest of the study groups.

Discussion

In the present study we have investigated the potential clinical performance of an IP-10 ELISA in-house for active TB and LTBI diagnosis in children recruited from three different study groups: active TB, LTBI screenings and contact-tracing studies. In addition, we have compared this potential biomarker with IFN-γ responses measured by QFN-G-IT and T-SPOT.TB. We have found that IP-10 assay has a comparable performance to QFN-G-IT and T-SPOT.TB for LTBI diagnosis. In addition, when QFN-G-IT and IP-10 assays were combined, the overall number of positive results increased from 38.3% in QFN-G-IT and 33.9% in

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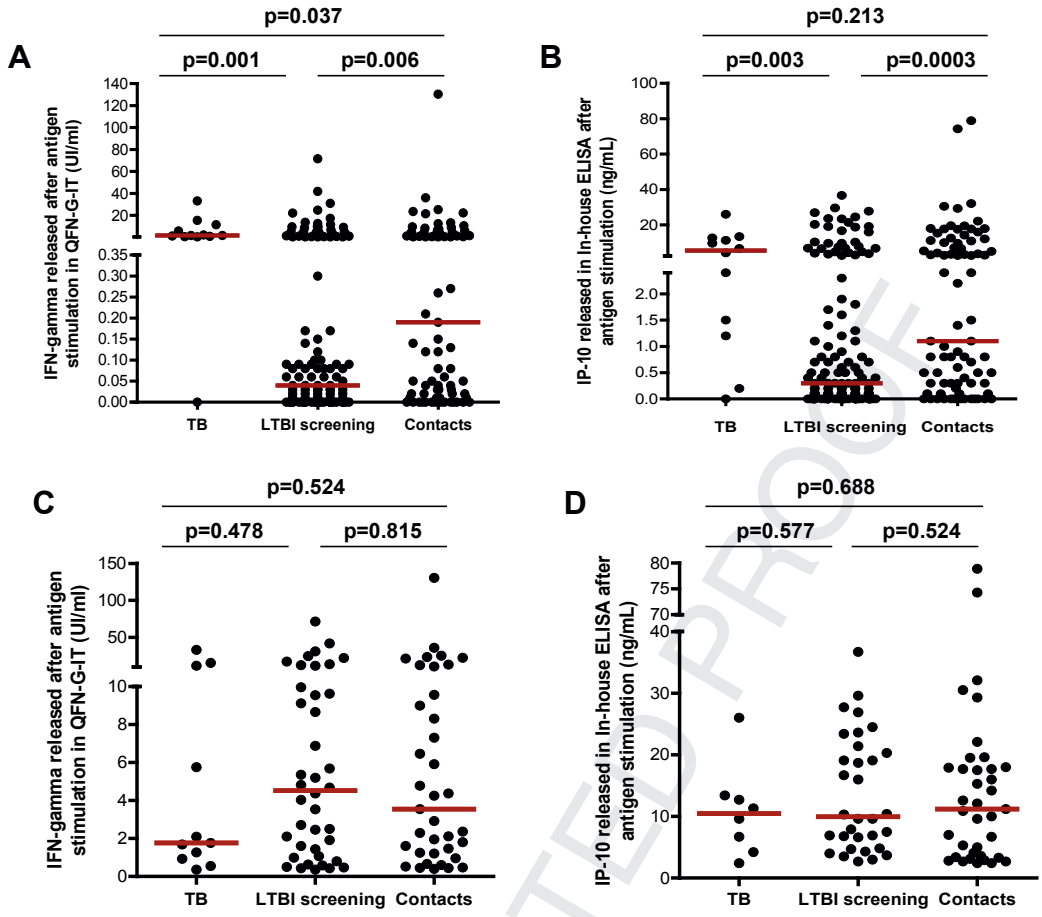

Figure 1 Amount of the cytokine released after MTB-specific antigen stimulation. Overall amount of IFN-γ(A) and IP-10 (B) released after overnight stimulation with ESAT-6, CFP-10 and TB7.7 specific antigens in all the children from the three study groups. IFN-γ(C) and IP-10 (D) released in only those children with a positive response for QFN-G-IT and IP-10 ELISA assay was also analysed. The median cytokine levels from each group are represented by the horizontal line. Statistical analysis was performed using Mann–Whitney test with Dunn-Bonferroni correction. *P*-value was considered significant if <0.016.

IP-10 test to 41.3%. Several authors have studied IFN-γ and IP-10 T-cell responses in paediatric population. In the majority of these studies, it has been demonstrated that IP-10 may represent a potential TB biomarker in this group of patients, and its combination with IFN-γ, when detected by QFN-G-IT, may improve IGRAs performance.[18,20,21,25–28] Nevertheless, available data is still limited and there is need of further research in this field. Additionally, the present study is the first one performing a direct comparison between T-SPOT.TB, QFN-G-IT and IP-10 ELISA In-house in children.

We observed that overall correlation between the three assays was good ($\kappa = 0.717–0.783$). In contrast, QFN-G-IT and IP-10 assays showed a weak correlation in the group of children with active TB ($\kappa = 0.308$). Lighter et al.,[18] measured IFN-γ levels by QFN and IP-10 levels by a microsphere-based assay in 127 children living in a region of low TB prevalence. In line with our results, they found a strong correlation of QFN with IP-10 detection, whereas discordant results were commonly found in children with active TB. The authors suggested that the combination of these two biomarkers in this group of children would help to improve the sensitivity for active TB diagnosis. In fact, it has been previously demonstrated that a combined IFN-γ/IP-10 biomarker approach can increase the sensitivity for diagnosing active TB in adults.[29]

The overall amount of IFN-γ and IP-10 released after antigen-specific stimulation in children with active TB was significantly higher in relation to all children enrolled during LTBI screenings. In contrast, when we compared cytokine secretion only in children with a positive response, we did not obtain any significant difference between active TB and LTBI groups. In fact, we have reported similar observations in a previous published manuscript.[30] This could be explained by the fact that paediatric infection is usually recent; as a consequence, immunological response is strong and equal with that observed in active TB. Comparable results have been described by other authors.[20,27]

We also analysed possible risk factors associated with positive QFN-G-IT and IP-10 results. In our study, none of the children included developed active TB. Age and type of contact were the two variables that showed significant association for both assays in the multivariate analysis. In this sense, prospective studies evaluating how these factors could affect positive predictive value for progression to active TB are urgently needed.

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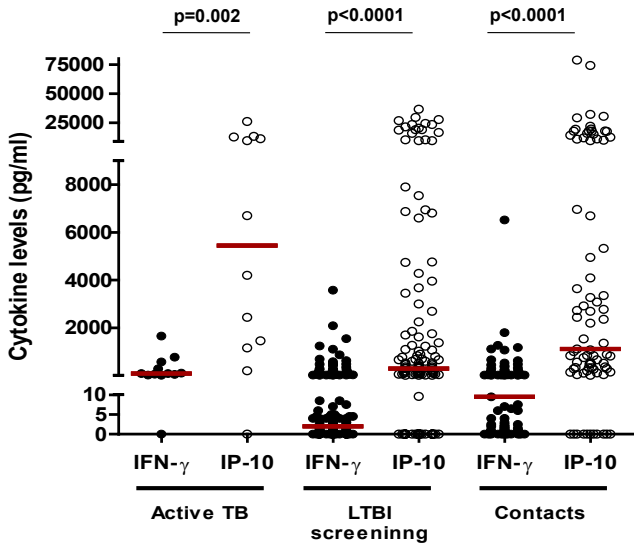

Figure 2 Comparison of IFN-γand IP-10 levels (pg/ml) after antigen-specific stimulation for the different study groups. The median cytokine levels from each group are represented by the horizontal line. Closed symbols represent IFN-γand open symbols IP-10. Statistical analysis was performed using Mann—Whitney analysis.

There is no gold standard assay for LTBI diagnosis. Consequently, one possible option for studying the sensitivity of T-cell based assays is their evaluation in active TB patients. As a result, obtained results did not reach 100% sensitivity.[8,31,32] In our study, sensitivity of both QFN-G-IT and T-SPOT.TB assays for active TB was 91.7% (11/12). In addition, sensitivity of IP-10 assay was 66.7% (8/12). One possible reason for these findings could be the possible role of $CD4^{+}CD25^{+}Foxp3^{+}$ regulatory T cells (Tregs) as essential players in dampening the effector immune response in active TB via programmed death-1 (PD-1) and IL-10 secretion.[33] Moreover, specificity of IP-10 has been demonstrated to be high in healthy controls.[18,25,29] In our study, we investigated IP-10 response in a subgroup of 21 TST negative healthy children recruited during LTBI screening group, enrolled as part of routine screenings at school. We observed that all these healthy children had negative responses for both cytokines, indicating that IP-10 specificity is high.

Lighter et al.[18] found no differences in IP-10 mitogen-stimulated levels between children at various ages. However, they observed that IFN-γ mitogen-stimulated levels were age-dependent. In the same way, Alsleben et al.[20] did not observed a direct association between IP-10 mitogen response and age. Similar results were also described by Kabeer et al..[21] On the contrary, Ruhwald et al.[25] found a significant age associated increase rate of positive responders for IP-10, but not for QFN-G-IT in a group of recently exposed children from Nigeria. In our study, the concentration of IFN-γ and IP-10 released after mitogen stimulation did not correlate with age. One possible explanation for this observation could be that the majority of the children were aged above 5 years old.

IP-10 cytokine is expressed at 100-fold higher amounts compared with IFN-γ.[11,24] In agreement with existing data, we have observed significant higher levels of IP-10 in comparison to IFN-γ after MTB specific-antigen stimulation in the different study groups.[18,20,26,27] It has been suggested that this high secretion of IP-10 cytokine could enable the use of smaller volumes of blood and a simple readout system.[13] In this direction, an IP-10 ELISA assay based on dried plasma spots (DPS) in filter paper has been developed. It has been demonstrated that this method had an equivalent diagnostic accuracy as QFN-G-IT and that samples can be sent via conventional mail over long distances.[23,24,34]

Quantification of IFN-γ and/or IP-10 in blood cannot distinguish between disease and infection. Several approaches measuring multiple cytokines have been investigated. It has been proposed that a dominant frequency of single-positive TNF-α-producing $CD4^{+}$ T cells detected by flow cytometry was associated to active TB.[35] In the same context, other authors have described that a frequency of PPD-specific IFN-γ/IL-2 dual-positive $CD4^{+}$ T cells below

Table 5 Distribution of QFN-G-IT, IP-10 and T-SPOT.TB results in the different study groups.

		QFN-G-IT	IP-10 test	T-SPOT.TB	T-SPOT.TB + IP-10
Overall	Positive	68 (37.6)	63 (34.8)	67 (37)	78 (43.1)*
	Negative	112 (61.9)	116 (64.1)	111 (61.3)	103 (56.9)
	Indeterminate	1 (0.6)	2 (1.1)	3 (1.7)	0 (0)
Active TB	Positive	11 (91.7)	8 (66.7)	11 (91.7)	12 (100)τ
($n = 12$)	Negative	1 (8.3)	4 (33.3)	0 (0)	0 (0)
	Indeterminate	0 (0)	0 (0)	1 (8.3)	0 (0)
Contact-tracing studies	Positive	36 (46.8)	36 (46.8)	36 (46.8)	41 (53.2)ŧ
($n = 77$)	Negative	41 (53.2)	41 (53.2)	40 (51.9)	36 (46.8)
	Indeterminate	0 (0)	0 (0)	1 (1.3)	0 (0)
LTBI screening	Positive	21 (22.8)	19 (20.6)	20 (21.7)	25 (27.2)Ψ
($n = 92$)	Negative	70 (76.1)	71 (77.2)	71 (77.2)	67 (72.8)
	Indeterminate	1 (1.1)	2 (2.2)	1 (1.1)	0 (0)

*$p < 0.002$ compared to IP-10 and T-SPOT.TB.
τ $p = 0.046$ compared to IP-10.
ŧ $p = 0.34$ compared to IP-10 and T-SPOT.TB.
Ψ $p = 0.005$ and $p = 0.034$ compared to IP-10 and T-SPOT.TB, respectively.

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Table 6 Positive/negative concordance score and agreement between QFN-G-IT, IP-10 test and T-SPOT.TB results.[a]

	QFN-G-IT vs IP-10 test				T-SPOT.TB vs IP-10 test				QFN-G-IT vs T-SPOT.TB			
	Pos[d]	Neg[d]	Agreement (%)	*K* (SE[b])	Pos[d]	Neg[d]	Agreement (%)	*K* (SE[b])	Pos[d]	Neg[d]	Agreement (%)	*K* (SE[b])
Overall	71	132	203/227 (89.4)	0.773 (0.044)	52	101	153/176 (86.9)	0.717 (0.055)	57	102	159/177 (89.8)	0.783 (0.048)
Active TB	8	1	9/12 (75)	0.308 (0.250)	7	0	7/11 (63.6)	—[c]	10	0	10/11 (90.9)	—[c]
Contact-tracing studies	34	40	74/81 (91.3)	0.826 (0.063)	31	36	67/76 (88.1)	0.762 (0.074)	31	36	67/76 (88.1)	0.762 (0.074)
LTBI screening	29	91	120/134 (89.6)	0.735 (0.066)	14	65	79/89 (88.8)	0.666 (0.097)	16	66	82/90 (91.1)	0.743 (0.086)

[a] Indeterminate results were not included in the analysis.
[b] Standard deviation.
[c] All T-SPOT.TB results were positive for this study group; therefore, κ value is not possible to calculate because a symmetrical table is required.
[d] Positive/negative concordance score.

56% was an accurate marker for active TB.[36] Besides, IFN-γ and IP-10 responses to novel TB antigens like RD1 selected peptides, DosR-regulon encoded proteins or resuscitation promoting factors have been recently described.[37–40] For example, Chegou et al.[39] found that Rv0081-specific levels of IL-12, IP-10, IL-10 and TNF-α were promising active TB diagnostic candidates. As well, Kabeer et al.[37] discovered that both IP-10 and IFN-γ responses to RD1 selected peptides could be useful biomarkers for monitoring the efficacy of therapy in patients with active TB.

The main drawbacks of our study have to be addressed. Given that there is no gold standard for LTBI, it is not possible to identify which of the discordant results is the right one. So, the combination of several assays that detect different cytokines could be favourable for high-risk individuals with an impaired immune system, like immunosuppressed patients or children aged below 5 years old. Furthermore, even though we have compared QFN-G-IT, an IP-10 ELISA in-house and T-SPOT.TB in the same paediatric population, the sample size of children with active TB is small. Nevertheless, the results reported here are reliable enough to demonstrate the potential role of IP-10 in TB diagnosis as an adjunct marker with IFN-γ.

In the present study, we have found that IP-10 cytokine is expressed in response to TB specific-antigens used in QFN-G-IT. In conclusion, the use of IFN-γ T-cell based assays in combination with an additional IP-10 assay detection could be a useful method for diagnosing active TB and LTBI in children. In addition, reducing the threshold cut offs could improve the potential diagnostic of these techniques in paediatric population. Taken together, these findings could open the attractive possibility of developing next generation immunodiagnostic assays based on new biomarkers detection, such as IP-10. However, further investigations are required to develop novel state-of-the-art techniques that could be transformed into point-of-care assays for TB diagnosis in high-risk individuals.

Acknowledgements

We thank the nurse staff of the Unidad de Prevención y Control de la Tuberculosis of Barcelona; and C. Ramil, L. Haba, M.A. Cuesta and M. Pérez for technical assistance. A native English speaker has revised the manuscript.

This work was supported by a grant from the Sociedad Española de Neumología y Cirugía Torácica (SEPAR); the Societat Catalana de Pneumologia (SOCAP) and the Fundació Catalana de Pneumologia (FUCAP).

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