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DIAGNOSTICS

A multicentre evaluation of the accuracy and performance of IP-10 for the diagnosis of infection with *M. tuberculosis*

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SUMMARY

IP-10 has potential as a diagnostic marker for infection with *Mycobacterium tuberculosis*, with comparable accuracy to QuantiFERON-TB Gold In-Tube test (QFT-IT). The aims were to assess the sensitivity and specificity of IP-10, and to evaluate the impact of co-morbidity on IP-10 and QFT-IT.

168 cases with active TB, 101 healthy controls and 175 non-TB patients were included. IP-10 and IFN-γ were measured in plasma of QFT-IT stimulated whole blood and analyzed using previously determined algorithms. A subgroup of 48 patients and 70 healthy controls was tested in parallel with T-SPOT.TB

IP-10 and QFT-IT had comparable accuracy. Sensitivity was 81% and 84% with a specificity of 97% and 100%, respectively. Combining IP-10 and QFT-IT improved sensitivity to 87% ($p < 0.0005$), with a specificity of 97%. T-SPOT.TB was more sensitive than QFT-IT, but not IP-10. Among non-TB patients IP-10 had a higher rate of positive responders (35% vs 27%, $p < 0.02$) and for both tests a positive response was associated with relevant risk factors. IFN-γ but not IP-10 responses to mitogen stimulation were reduced in patients with TB and non-TB infection.

This study confirms and validates previous findings and adds substance to IP-10 as a novel diagnostic marker for infection with *M. tuberculosis*. IP-10 appeared less influenced by infections other than TB; further studies are needed to test the clinical impact of these findings.

1. Introduction

IFN-γ release assays (IGRAs) are an upgrade to the century old tuberculin skin test (TST). The IGRAs utilize T cell recognition of *Mycobacterium tuberculosis*-specific peptides and are therefore almost exclusively positive in patients infected with the bacteria belonging to the *M. tuberculosis* complex. In contrast to the IGRAs, the TST cross-reacts with the BCG vaccine, therefore the IGRAs are more accurate in vaccinated individuals, and are rapidly becoming the test-of-choice when screening exposed and infected individuals at risk of progression to active TB in high resource settings.[1,2]

The sensitivity of IGRAs is suboptimal. Using confirmed active TB disease as a reference standard for *M. tuberculosis* infection, the

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most recent meta-analysis suggests that the most commonly used IGRA – the QuantiFERON-TB Gold In-Tube test (QFT-IT) – detects approximately 80% (95% CI: 75–84%) of infected individuals[3] and has a specificity of 99% (95% CI: 97.9–99.9%).[4] The IGRAs have a high negative prognostic value for overt TB disease in low endemic regions,[4–6] whereas the positive and negative predictive values for progression to active TB disease in medium and high endemic regions appear less pronounced.[7–9]

In our opinion the shortcomings of the IGRAs could be due to the features of IFN-γ as readout biomarker.[10] IFN-γ is a cytokine expressed at low levels, close to the detection limit of the assays, e.g. 17.5 pg/ml (0.35 IU/ml) for the QFT-IT.[11,12] Alternative biomarkers expressed in higher magnitude may enable a more sensitive test that could detect weak antigen-specific responses that remain undetected with IFN-γ.

We have screened a large panel of potential biomarker candidates[13–15] of which the monocyte derived chemokine IP-10 has shown most promise. In a cohort of 124 controls and 86 patients with active TB, we have established a cut-off for a positive IP-10 test using Luminex,[16] and demonstrated that the IP-10 test performs at least as accurately as the QFT-IT in a clinical study of exposed children[17] and in HIV-infected adults with active TB.[45] Other groups have shown similar results in both adults[18] and children,[19,20] and two recent studies in HIV-infected adults have demonstrated that IP-10 detected a greater number of HIV–TB cases than IFN-γ and suggested that IP-10 could be a better alternative marker for diagnosing latent TB infection among immuno-compromised individuals.[21,45] A limitation of the previous studies is that estimates of sensitivity and specificity were done based on the same cohort used to determine the optimal cut-off.

Although IGRAs are not marketed for the diagnosis of active TB, they are frequently applied as one of several diagnostic tools in suspected patients. Because IGRAs do not discriminate between active and latent TB infection, their usefulness for diagnosing active TB is likely limited in a clinical setting where patients have a high pre-test probability of latent infection.[22]

The present study aimed to investigate two questions. The first was to determine the sensitivity and specificity of the previously developed IP-10 test, to validate its cut-off. For this part of the study we included patients with confirmed TB and healthy presumed unexposed controls. The second question addressed the impact of other diseases on the diagnostic performance. For this part of the study we included a group of sick patients who were suspected of active TB disease, but who received another final diagnosis.

2. Material and methods

2.1. Study population

Patients with active TB (TB), healthy controls (HC) and non-TB patients (non-TB) were included from 9 centres in Europe affiliated to TBNET (Table 1). Except for the Perugia centre that included patients prospectively, TB and non-TB samples were included retrospectively from the participating centres biobanks. Samples are routinely stored in the biobanks to enable confirmation of test results, for quality control issues or for future clinical studies like this. Patient groups were defined based on the following criteria. Active TB: confirmed TB based on positive culture, positive PCR; and/or positive microscopy or histology and a response to treatment. Non-TB patients: sick adults who were initially suspected of active TB, but who ended up not having active TB but other diagnoses (e.g. pneumonia or lung cancer). Non-TB patients were defined by the following criteria: negative microbiological investigations for TB and either a confirmed alternative diagnosis explaining the condition and response to relevant treatment or a confirmed chronic condition such as cancer, or recovery without anti-TB treatment. Demographic and clinical data was collected from patient files. Immuno-suppression was classified according to Lee et al.[23] All patients were IGRA tested as part of the diagnostic procedure in patients with clinical suspicion of TB, among the TB patients blood for IGRA was drawn within the first two weeks of anti-TB treatment. The healthy controls comprised students from a high school in the greater Copenhagen area, Denmark[24] and from the School of Medicine at the University of Modena and Reggio Emilia, all controls had no known exposure to *M. tuberculosis* and no prior TB diagnosis or treatment. We furthermore included samples from non-exposed volunteers among the staff at the Copenhagen and Barcelona centres. Neither IGRA, IP-10 nor TST results were used to define the three groups. The relevant ethical committees at each centre approved the study protocol.

Table 1
Overview of centres and participants.

	All (EU)	Modena (I)	Perugia (I)	Rome (I)	Copenhagen (DK)	Stockholm (S)	Barcelona (Sp)	Thessaloniki (Gr)	Terni (I)	Helsinki (Fi)
N	444	149	89	46	41	39	36	27	9	8
Age median (range)	38 (18–92)	28 (18–91)	53 (18–92)	36 (19–81)	18 (18–68)	41 (19-83)	34 (18–85)	56 (23–89)	51 (18–77)	58 (38–64)
Male sex, *n*(%)	263 (59)	97 (66)	52 (58)	29 (63)	16 (39)	21 (54)	23 (64)	16 (59)	4 (44)	5 (63)
Diagnosis										
Tuberculosis										
Pulmonary, *n*(%)	130 (77)	37 (80)	13 (52)	34 (94)	1 (100)	4 (31)	30 (100)	10 (63)	1 (100)	0 (0)
Extrapulmonary, *n*(%)	30 (18)	7 (15)	9 (36)	0 (0)	0 (0)	8 (61)	0 (0)	6 (37)	0 (0)	0 (0)
Pulmonary and extrapulmonary, *n*(%)	8 (5)	2 (4)	3 (12)	2 (6)	0 (0)	1 (8)	0 (0)	0 (0)	0 (0)	0 (0)
Non-TB patients										
Cancer, *n*(%)	13 (7)	0 (0)	5 (8)	0 (0)	1 (9)	1 (4)	0 (0)	3 (27)	1 (13)	2 (25)
Infection, *n*(%)	120 (69)	37 (100)	49 (77)	6 (60)	6 (55)	9 (35)	0 (0)	5 (45)	6 (75)	2 (25)
Autoimmune disease, *n*(%)	5 (3)	0 (0)	3 (5)	0 (0)	0 (0)	1 (4)	0 (0)	0 (0)	0 (0)	1 (13)
Other, *n*(%)	17 (10)	0 (0)	7 (11)	2 (20)	1 (9)	3 (12)	0 (0)	0 (0)	1 (13)	3 (38)
Unknown, *n*(%)	20 (11)	0 (0)	0 (0)	2 (20)	3 (27)	12 (46)	0 (0)	3 (27)	0 (0)	0 (0)
Healthy controls	101 (100)	66 (100)	0 (0)	0 (0)	29 (100)	0 (0)	6 (100)	0 (0)	0 (0)	0 (0)

2.2. IP-10 and IFN-γ measurements and test interpretation

The QFT-IT (Cellestis, Carnegie, Australia) and the T-SPOT.TB (Oxford Immunotec, Abingdon, United Kingdom) tests were done in accordance to manufacturer's instructions at the participating centres. After QFT-IT testing, the QFT-IT supernatants were frozen. IP-10 in samples from Barcelona was analyzed on site whereas; samples from the other sites were shipped to Copenhagen and measured there. All samples were run in duplicate by xMAP/Luminex technology as described previously.[16] The same type of hardware, software and batch of IP-10 assay was used at the two sites. The antigen-dependent and mitogen-induced biomarker production were measured by subtracting the concentration measured in the nil. The antigen-dependent and mitogen-induced levels of IP-10 were reduced to positive, negative and indeterminate test outcome using an algorithm previously defined on a cohort of TB patients and healthy controls using ROC curve analysis. The cut-off for positive IP-10 test was 673 pg/ml and for indeterminate IP-10 test was 200 pg/ml.[16] QFT-IT tests were analyzed and interpreted in accordance to manufacturer's instructions. T-SPOT.TB results were analyzed in accordance with the European T-SPOT.TB interpretation algorithm, (≥6 spots in either panel A or B after subtracting the number in the Nil panel was considered positive).

2.3. Data analysis

Data were analyzed using SAS 9.2 (SAS institute, USA). Variables were compared using non-parametric tests where appropriate. Estimates of sensitivity and specificity are presented after excluding indeterminate responders. All tests were two sided and p-values <0.05 were considered significant. For further information on patient inclusion and data analysis refers to Supplementary data file.

3. Results

A total of 168 patients with TB, 101 HC and 175 non-TB patients were included in the study (Table 1). Among the TB patients, 77% (130/168) had pulmonary disease, and 91% (143 of 157 tested) were culture or PCR confirmed (Table 2). Seven percent (11 of 157) TB patients with known HIV status were HIV-infected, and 13% (22 of 166) had another immuno-suppressant condition. The HCs were all from Western Europe, 67% (66/101) were from Italy, 6% (6/101) from Spain and 29% (29/101) were from Denmark. HCs were significantly younger than non-TB and TB patients ($p < 0.0001$). Among the non-TB patients, 69% (120/175) had bacterial (88%) or viral (12%) infection, 10% (17/175) cancer, 3% (5/175) inflammatory diseases, 10% (17/175) had other diseases, and for 11% (20/175) the

Table 2
Baseline table.

		TB patients	Healthy controls	Non-TB patients
N		168	101	175
Age median (range)		37 (18–90)	22 (18–53)	56 (18–92)
Male sex, *n*(%)		96 (57)	60 (59)	107 (61)
Region of birth, *n*(%)	Western Europe	59 (39)	101 (100)	125 (71)
	Eastern Europe	31 (21)	0 (0)	9 (5)
	Africa	27 (18)	0 (0)	27 (15)
	Asia	24 (16)	0 (0)	10 (6)
	South America	9 (6)	0 (0)	4 (2)
Severity, *n*(%)	Outpatient	16 (10)	–	71 (41)
	Required admission	122 (73)	–	102 (58)
	Unknown	30 (18)	–	2 (1)
Immuno-suppression				
HIV status, *n*(%)	Positive	11(7)	1 (1)	26 (15)
	Negative	146 (87)	71 (70)	125 (71)
	Unknown	11 (7)	29 (29)	24 (14)
Other immuno-suppression, *n*(%)	Yes	22 (13)	0 (0)	34 (19)
	No	144 (86)	94 (93)	97 (55)
	Unknown	2 (1)	7 (7)	44 (25)
TB risk factors				
Prior TB, *n*(%)	Prior TB	12 (7)	0 (0)	16 (9)
	No prior TB	114 (68)	101 (100)	139 (79)
	Unknown prior TB	42 (25)	0 (0)	20 (12)
Exposure, *n*(%)	TB exposure	19 (11)	0 (0)	15 (9)
	No exposure	96 (57)	101 (100)	124 (71)
	Unknown exposure	53 (32)	0 (0)	36 (20)
Stay in TB endemic country >2 months, *n*(%)	Yes	69 (41)	0 (0)	44 (25)
	No	47 (28)	97 (96)	111 (63)
	Unknown	52 (31)	4 (4)	20 (12)
TB diagnostic tests				
Culture, *n*(%)	Positive	140 (83)	–	0 (0)
	Negative	13 (8)	–	95 (54)
	Not done	1 (1)	–	20 (11)
	Not available	14 (8)	–	60 (34)
PCR, *n*(%)	Positive	65 (39)	–	0 (0)
	Negative	33 (20)	–	88 (50)
	Not done	13 (8)	–	23 (13)
	Not available	57 (34)	–	64 (37)
Microscopy, *n*(%)	Positive	73 (43)	–	0 (0)
	Negative	81 (48)	–	95 (54)
	Not done	1 (1)	–	21 (12)
	Not available	13 (8)	–	59 (34)

final diagnosis was unknown, and the patients recovered without anti-TB treatment. Sixty percent (102/173) of the non-TB patients were admitted to hospital; 35% (61/175) had at least one risk factor for TB infection; 17% (26/151) with known HIV status were HIV-infected; and 10% (16/155) with available information reported prior TB disease.

3.1. Biomarker levels

Biomarker levels are described in detail in Table 3. The nil IP-10 and IFN-γ levels were significantly lower in HCs compared to TB and non-TB patients, although the differences for IFN-γ were very small. TB patients produced significantly higher absolute levels of antigen-induced IP-10 and IFN-γ compared to the other groups, and non-TB patients produced higher levels than the healthy controls. In paired comparisons, TB patients produced antigen-induced IP-10 in median 29.1 (IQR 10.5–60.0) fold higher magnitude compared with IFN-γ ($p < 0.0001$), and the median signal-to-noise ratio (antigen-induced divided with nil level) for IFN-γ was 25.3 (IQR 5.5–70.0) compared with 19.0 (IQR 4.1–56.7) for IP-10 ($p = 0.07$). Mitogen-induced IFN-γ responses were significantly reduced in TB patients compared to HC ($p < 0.05$) and in non-TB patients compared to HC ($p = 0.0002$) whereas the IP-10 responses were not reduced in neither patients with active TB or in non-TB patients. To investigate these differences further we divided the group of non-TB patients into patients with bacterial and viral infection ($n = 120$) and patients with other known diseases (e.g. cancer and autoimmune diseases $n = 35$) and compared responses to healthy controls as reference (Figure 1). The subgroup with bacterial or viral infection had significantly lower levels of mitogen-induced IFN-γ (median 101 pg/ml vs 460 pg/ml, $p < 0.0001$), whereas the mitogen-induced levels were not affected in the group of non-TB patients with other diseases (median 415 pg/ml, $p = 0.823$). In contrast, mitogen-induced IP-10 in infected non-TB patients appeared less and not significantly affected compared to healthy controls (median 1741 pg/ml vs 2216 pg/ml, $p = 0.064$) and the uninfected non-TB patients had a median of 3384 pg/ml, $p = 0.078$. There was no effect on these differences when excluding HIV-infected patients and the impact of infection was not related to viral or bacterial pathogen (data not shown).

3.2. IP-10 and IFN-γ test performance: sensitivity, specificity and indeterminate rate

The TB patients and HC were used to compare the diagnostic accuracy of IP-10 and IFN-γ. IP-10 had a high diagnostic accuracy with an AUC of 0.924 comparable to that of IFN-γ 0.937 ($p = 0.89$, graphs not shown). The specificity of the IP-10 test was 97% at 81% sensitivity; 2% had indeterminate responses (Table 4). In comparison QFT-IT specificity was 100%, at 84% sensitivity; 4% were QFT-IT indeterminate.

The concordance between the IP-10 test and QFT-IT was substantial; both tests were positive in 123/168 (73%), and negative in 18/168 (11%) of the TB patients. Agreement was 97/101 (96%, $k > 0.39$) among healthy controls and 143/168 (85%, $k = 0.57$) among TB patients. When combining the IP-10 test and QFT-IT tests, the sensitivity increased significantly to 145/166 (87%) ($p = 0.005$) without a compromise in specificity (97/100) 97% (Table 4). Twenty-five TB patients had discordant results. Nine were QFT-IT positive and IP-10 test negative, of which three had an immuno-suppressant co-morbid condition. Eight were QFT-IT negative and IP-10 test positive of which one had an immuno-suppressant co-morbid condition. Five patients were IP-10 test negative, QFT-IT indeterminate of which 3 had an immuno-suppressant condition.

3.3. QFT-IT, IP-10 and T-SPOT.TB results

T-SPOT.TB results were available in a subgroup of 48/168 (29%) TB patients and in 70/101 (70%) of the healthy controls (Table 5). IP-10 and T-SPOT.TB detected more patients as positive compared to QFT-IT in this subgroup (41/48 (85%) and 43/48 (90%) vs 37/48 (77%), $p < 0.05$ and $p < 0.04$ respectively), and there were no significant differences between T-SPOT.TB and IP-10 ($p = 0.32$). Of the 7 patients that were T-SPOT.TB positive QFT-IT negative IP-10 detected 4. The 3 T-SPOT.TB positive IP-10 negative TB patients had 36, 17, 9 and 7, 0, 54 spots in A and B panel, respectively and the median number of spots in the T-SPOT.TB and IP-10 concordant positive responders was 42 (IQR 15–90) and 75 (IQR 23–132) for A and B panel, respectively. One patient was QFT-IT positive T-SPOT.TB negative, this patient was also IP-10 positive. There were no significant difference in the rate of negative responders among the controls IP-10:68/70 (97%), T-SPOT.TB: 69/70 (99%) and QFT-IT 70/70 (100%), $p > 0.32$. The control with positive T-SPOT.TB had 17 and 192 spots in the ESAT6 and CFP10 wells, respectively. Combining IP-10 and QFT-IT improved QFT-IT sensitivity significantly ($p < 0.05$), but IP-10 did not have added value when combined with T-SPOT.TB ($p = 0.32$).

3.4. Test performance in non-TB patients

Although IGRAs are not marketed for the diagnosis of active TB, they are frequently applied as one of several diagnostic tools in patients suspected of active TB.[18,25–28] In order to identify potential effects of non-TB diseases on the test performance, we included a heterogeneous group of patients who had been suspected of TB but where TB was excluded and other diagnoses were found. In this group the IP-10 test was positive in 61 (35%) and QFT-IT in 47 (27%), $p < 0.02$. Fourteen percent (24/175) had an indeterminate QFT-IT result and 9% (16/175) an indeterminate IP-10 test ($p = 0.054$), (Table 4). Agreement was 73% (128/175, $k = 0.52$), 46% (81/175) were concordant negative, 22% (39/175) were concordant positive. When combining the IP-10 test and the QFT-IT the number of positive responders increased to 39% (69/175). In order to evaluate whether positive test results among non-TB patients were associated with risk factors for *M. tuberculosis* infection, we calculated the age and sex adjusted Odds Ratio (OR) for positive tests (Table 6). The IP-10 test was significantly increased among patients born in a high endemic region (OR 4.0) and in patients with a prior TB

Table 3
Biomarker levels (pg/ml), median (inter quartile range), Kruskal–Wallis test.

		TB patients	Healthy controls	Non-TB patients	HC vs TB	*p*-values HC vs non-TB	Non-TB vs TB
IFN-γ	Nil	7 (4–16)	5 (3–8)	5 (2–9)	0.0047	0.5179	0.0002
	Antigen-dependent	136 (25–393)	0 (0–1)	0 (0–20)	<0.0001	<0.0001	<0.0001
	Mitogen-induced	391 (75–492)	460 (271–492)	200 (41–492)	0.0487	0.0002	0.0984
IP-10	Nil	207 (120–442)	99 (53–239)	152 (77–329)	<0.0001	0.0337	0.0015
	Antigen-dependent	3414 (873–10,547)	20 (0–79)	182 (13–1496)	<0.0001	<0.0001	<0.0001
	Mitogen-induced	2680 (880–7563)	2216 (1182–5062)	2193 (903–4916)	0.4101	0.5307	0.0771

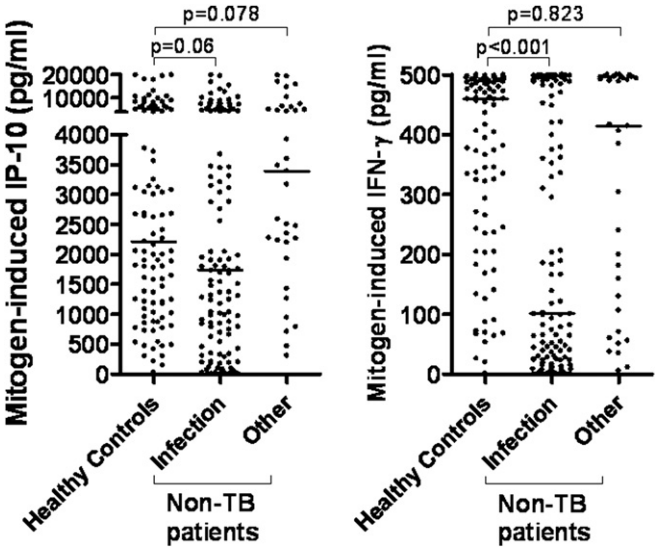

Figure 1. The distribution of mitogen-induced (mitogen subtracted nil) IP-10 and IFN-γ responses in healthy controls and non-TB patients divided into the group with infection ($n = 120$) and a group with known other final diagnosis (e.g. cancer, autoimmune diseases, sarcoidosis ($n = 35$)), Solid lines denote median; significance level was assessed using Kruskal–Wallis test.

diagnosis (OR 5.9). The QFT-IT was related to the same risk factors; ORs for a positive test were 3.1 and 5.0, respectively. Neither test was associated with a history of prior exposure to a TB patient (OR 2.9), nor prolonged stay in a TB endemic area (OR < 2.1). The OR for the combined IP-10 test and QFT-IT test was higher than IP-10 test alone in patients with prior TB and in patients with history of exposure, but lower among patients born in a TB high endemic country (data not shown).

3.5. Indeterminate responders among the non-TB patients

Fourteen percent had an indeterminate QFT-IT result and 9% had an indeterminate IP-10 test ($p = 0.054$). Among the non-TB patients with a viral or bacterial infection, 13% (16/120) were IP-10 test indeterminate in contrast to none of those with other diseases ($p = 0.01$), for QFT-IT 18% (22/120) were indeterminate in contrast to 6% (2/35) ($p = 0.041$), respectively. In addition 6% (10/175) were treated with corticosteroids of which 20% (2/10) were IP-10 test positive/QFT-IT indeterminate and 10% (1/10) were concordant indeterminate. Among the discordant responders 73% (22/30) were IP-10 test positive QFT-IT negative ($n = 17$) or indeterminate ($n = 5$), and 40% (8/30) were QFT-IT positive IP-10 test negative ($n = 6$) or indeterminate ($n = 2$).

Table 4
Distribution of positive, negative and indeterminate responders with the IP-10 test and QFT-IT and when the tests are combined.

		IP-10 test	QFT-IT	IP-10 test + QFT-IT
TB patients, *n*(%)	+	133 (79)	133 (79)	145 (86)*,†
$N = 168$	−	32 (19)	26 (15)	21 (13)
	Indet	3 (2)	9 (5)	2 (1)
Healthy controls, *n*(%)	+	3 (3)	0 (0)	3 (3)
$N = 101$	−	96 (95)	100 (99)	97 (96)
	Indet	2 (2)	1 (1)	1 (1)
Non-TB patients, *n*(%)	+	61 (35)‡	47 (27)	69 (39)*
$N = 175$	−	98 (56)	104 (59)	97 (55)
	indet.	16 (9)	24 (14)	9 (5)

* $p < 0.0005$ compared to QFT-IT.
† $p < 0.0005$ compared to IP-10 test.
‡ $p < 0.02$ compared to QFT-IT.

Table 5
A three-way comparison of IP-10 QFT-IT and T-SPOT.TB test results in a subgroup of 48 patients and 70 controls.

	N(%)	IP-10	QFT-IT	T-SPOT.TB
Patients	36 (75)	Positive	Positive	Positive
	4 (8)	Positive	Negative	Positive
	1 (2)	Positive	Positive	Negative
	1 (2)	Negative	Indeterminate	Positive
	1 (2)	Negative	Indeterminate	Indeterminate
	2 (4)	Negative	Negative	Positive
	1 (2)	Negative	Negative	Indeterminate
	2 (4)	Negative	Negative	Negative
Controls	67 (96)	Negative	Negative	Negative
	1 (1)	Negative	Negative	Positive
	2 (3)	Positive	Negative	Negative

4. Discussion

We report here the results of a comparative evaluation of IP-10 and IFN-γ as biomarkers in diagnostic tests for infection with *M. tuberculosis*. To evaluate sensitivity and specificity we included patients with confirmed TB and healthy presumed unexposed controls, and to evaluate performance in a clinical setting we included a group of patients who were suspected of active TB disease, but where another diagnosis was found. The diagnostic accuracy of IP-10 measured with Luminex was evaluated using a previously determined algorithm and cut-offs.[16]

We obtained two important results. First, we confirmed previous findings that IP-10 is produced in high amounts in stimulated whole blood from TB patients but not from HCs. This gives strength and robustness to IP-10 and to the algorithm we have previously set. Inline with our previous study using the same commercially available Luminex assay,[16] we found that the IP-10 test had comparable performance to QFT-IT and T-SPOT.TB, and that QFT-IT and IP-10 tests could be combined for a significant improvement in sensitivity without a compromise in specificity.

We found a higher proportion of IP-10 positive non-TB patients compared to QFT-IT. Due to the lack of a reference for LTBI we do not know if these patients had LTBI and if IP-10 was more sensitive for LTBI than QFT-IT. The IP-10 positive non-TB patients did however have relevant risk for latent infection suggesting that they were correctly classified with LTBI. We found that the 3 IP-10 positive healthy controls were Italian students from Modena. They did not have any known risk factors for *M. tuberculosis* infection but all produced intermediate to high levels of antigen-dependent IP-10 (1300–2437 pg/ml) which suggests that the responses reflect the presence of a latent infection and not an unspecific signal around the cut-off.[29,30] In contrast the antigen-dependent IFN-γ levels were low in all three students when measured in the QFT-IT. In the subgroup of 70 students with T-SPOT.TB results available one student was T-SPOT.TB positive. Previously the specificity of the IP-10 test has been shown to be almost 100% among healthy subjects in low endemic regions.[16,18,19,31] It can be speculated that IP-10 picks up specific signals from individuals with a well controlled/resolved infection.[32] However, in the absence of a gold standard for TB infection, it remains to be demonstrated whether IP-10 is more sensitive for infection with *M. tuberculosis* or less specific than QFT-IT in otherwise healthy controls and in non-TB patients, and prospective studies are needed to evaluate how these differences affect the predictive values for progression to active TB in e.g. recently exposed with a positive test.

Our second important finding was the difference between the two biomarkers in the group of non-TB patients, where IP-10 release seemed less affected by bacterial or viral infection. Little is known about the influence of ongoing infectious disease and immune-

Table 6
The association between risk factors relevant to *M. tuberculosis* infection and test result in non-TB patients. Patients with missing information on risk factors and patients with an indeterminate IP-10 or QFT-IT response were excluded from the respective analysis. Odds ratios were adjusted for sex and age.

	n	IP-10 positive	AOR (95% CI)	*p*-value	*n*	QFT-IT positive	AOR (95% CI)	*p*-value
Born in TB endemic area	159				151			
Yes	49	27 (55)	4.0 (1.7–9.0)	<0.002	45	20 (44)	3.1 (1.3–7.3)	<0.009
No	110	34 (31)	1		106	27 (25)	1	
Exposure	126				119			
Yes	15	9 (60)	2.9 (0.9–9.2)	0.074	14	7 (50)	2.9 (0.9–9.6)	0.087
No	111	41 (37)	1		105	29 (28)	1	
Prior TB	140				132			
Yes	16	12 (75)	5.9 (1.8–20.0)	<0.004	14	9 (64)	5.0 (1.5–16.2)	<0.008
No	124	42 (34)	1		118	31 (26)	1	
Stay in high endemic area >3 months	140				132			
Yes	42	20 (48)	2.1 (0.9–5.0)	0.077	39	14 (36)	1.6 (0.6–3.8)	0.332
No	98	33 (34)	1		93	26 (28)	1	
>1 risk factor	124					116		
Yes	36	21 (58)	4.2 (1.7–10.8)	<0.003	33	15 (46)	3.5 (1.3–9.3)	<0.02
No	88	28 (32)	1		83	20 (24)	1	

suppression on IGRA performance in sick patients without active TB.[25,26,33] We and others have recently shown that HIV-infected individuals have a decline in IFN-γ responsiveness to mitogen and in QFT-IT sensitivity in patients with a low CD4 count, although IP-10 was influenced by HIV infection it was in a CD4 independent manner.[21,45] Young children (<5 years) are another important clinical challenge where the IGRAs – especially the QFT-IT – have shown compromised performance[34–36] two studies have indicated that IP-10 performs better in young children with an immature immune system.[17,19] These findings together with results presented here suggest that IP-10 could add diagnostic information in patients with immuno-suppression.

Our findings suggest a different interference of non-TB bacterial or viral infection on the performance of the two tests. Some of the disparities could be explained by the different cellular origin of the biomarkers and their very different role during infection. During severe infection, systemic immune responses coincide with counter-regulatory anti-inflammatory responses and changes in leukocyte number, function and phenotype (reviewed in Ref. [37]). These changes are also reflected in a decreased *ex-vivo* T cell responsiveness of pro-inflammatory cytokines (e.g. IL-2, IFN-γ), inline with our findings on IFN-γ mitogen responsiveness and QFT-IT performance. Monocyte function is also affected, both up- and down regulation of cytokine and chemokine responsiveness may occur.[38–41] The immunological mechanisms underlying these differences in IFN-γ and IP-10 are not fully understood, but they are likely to be attributed to the fact that IFN-γ is a cytokine produced by specific T cells when stimulated by the interaction with an antigen presenting cell (APC); whereas IP-10 is elicited in the APCs by signals from a range of cytokines (IL-1, IL-2, IFN-γ, IFN-α, TNF-α) combined with receptor mediated signals from adjacent T cells. And, as IP-10 is not exclusively dependent on IFN-γ expression, IP-10 can be induced by also by non-IFN-γ producing T cell sub-populations, and potentially lead to a more sensitive measure of T cell recognition.[42–44] Further studies are needed to elucidate the background for these discrepancies and to identify areas of potential synergy.

5. Limitations

The group of non-TB patients was typical cases suspected of active TB seen at both outpatients' clinics and among admitted patients, and is a very heterogeneous group. Apart from the Perugia centre, these patients were not prospectively included which reserved us from drawing conclusions on predictive values. The group of TB suspected non-TB patients however was thoroughly investigated for TB and in 72% of 116 with available information, either PCR or culture for *M. tuberculosis* was done and found negative, therefore we do consider this group to be a relevant control group for evaluating diagnostic tests. The applied IP-10 mitogen cut-off was arbitrarily set and has previously been tested in other studies. Compared to QFT-IT the IP-10 test mitogen cut-off is rather low in respect to the antigen responses, which reflects that the PHA mitogen acts directly on the T cells, whereas IP-10 responses are induced in the monocytes upon stimulation from T cell cytokines in the supernatant. Previously the 200 pg/ml cut-off resulted in a comparable rate of indeterminate responders compared to QFT-IT,[16] but in this study we found significantly less indeterminate IP-10 test results than the QFT-IT. Increasing the mitogen cut-off to e.g. 400 pg/ml resulted in higher rate of indeterminate responders 7/168 (4%), 21/175 (12%) and 4/103(4%) among TB patients, non-TB patients and HCs, respectively. These patients and controls converted from negative to indeterminate and lead to improved concordance with QFT-IT, and higher association between risk factors and positive tests in the non-TB group (data not shown). Further studies are needed to validate the cut-off for indeterminate IP-10 test.

The IP-10 measurements in this study have been acquired using Luminex, a technology that allows quantification of up to 200 different markers in a single 50 μL sample. This versatility comes at a price and new data from our group (unpublished) and by others[18,19,21,31] suggests that IP-10 measured with simpler ELISA technology leads to better reproducibility of the measurements, larger differences between nil and antigen responses and herewith better diagnostic accuracy.

6. Conclusion

In conclusion, this study confirms and validates antigen-specific IP-10 response as a diagnostic marker for infection with *M. tuberculosis* with comparable sensitivity and specificity to the QFT-IT and the T-SPOT.TB. IP-10 detected more non-TB patients as positive, although IP-10 responses were associated with relevant risk factors of TB infection it remains unanswered whether this was due to higher sensitivity or lower specificity.

IP-10 seems more robust due to the confirmed magnitude of IP-10 responses and appears less affected by non-TB bacterial or viral infection. The discrepancy between IFN-γ and IP-10 biomarkers needs further detailed characterisation and the potential

consequence of these differences in clinical practice prompts further investigation.

Perspectives

The high magnitude of IP-10 suggests that it can be measured with simpler technology e.g. the lateral flow platform known from pregnancy and HIV quick tests. The development of such a device-platform could enable the dissemination of specific tests for TB infection in low resource settings.

Authors' contributions

MR designed the study, included study participants, measured IP-10 and IFN-γ (ELISA), managed the master database, performed the statistics and drafted the manuscript; JD, IL included study participants, compiled clinical information and measured IFN-γ (ELISA and ELISPOT) and IP-10; ML, LR, included study participants, compiled clinical information and measured IFN-γ (ELISA and ELISPOT); MBP, RM, DG, OB, JB, HG, IG, TT and GF included study participants, compiled clinical information and measured IFN-γ (ELISA). JEO supervised the lab work and data interpretation, PR co-designed the study, included study participants, participated in the analysis of the results and helped to draft the manuscript. All authors played a part in drafting the final version of the manuscript and all approved the final version.

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Conflict of interest statement: Morten Ruhwald, Pernille Ravn and Jesper Eugen-Olsen are registered as inventors on patents filed by Hvidovre Hospital disclosing IP-10 as a diagnostic marker for infection with *M. tuberculosis*.

Appendix. Supplementary data

Supplementary data related to this article can be found online at doi:10.1016/j.tube.2011.01.001.

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