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# Evaluating the potential of IP-10 and MCP-2 as biomarkers for the diagnosis of tuberculosis

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**ABSTRACT: The aim of the present study was to evaluate the potential of diagnostic tests based on interferon-γ inducible protein (IP)-10 and monocyte chemotactic protein (MCP)-2, and compare the performance with the QuantiFERON TB® Gold In-Tube (QFT-IT; Cellestis, Carnagie, Australia) test.**

**IP-10 and MCP-2 were determined in supernatants from whole blood stimulated with *Mycobacterium tuberculosis*-specific antigens. Samples were obtained from 80 patients with culture- and/or PCR-proven tuberculosis (TB), and 124 unexposed healthy controls: 86 high school students and 38 high school staff. IP-10 and MCP-2 test cut-offs were established based on receiver operating characteristic curve analysis.**

**TB patients produced significantly higher levels (median) of IP-10 (2158 pg·mL$^{-1}$) and MCP-2 (379 pg·mL$^{-1}$) compared with interferon (IFN)-γ (215 pg·mL$^{-1}$). The QFT-IT, IP-10 and MCP-2 tests detected 81, 83 and 71% of the TB patients; 0, 3 and 0% of the high school students and 0, 16 and 3% of the staff, respectively. Agreement between tests was high (>89%). By combining IP-10 and IFN-γ tests, the detection rate increased among TB patients to 90% without a significant increase in positive responders among the students.**

**In conclusion, interferon-γ inducible protein-10 and monocyte chemotactic protein-2 responses to *Mycobacterium tuberculosis*-specific antigens could be used to diagnose infection. Combining interferon-γ inducible protein-10 and interferon-γ may be a simple approach to increase the detection rate of the *Mycobacterium tuberculosis*-specific *in vitro* tests.**

**KEYWORDS: Diagnosis, interferon-γ, interferon-γ release assay, tuberculosis, whole blood**

A major breakthrough in the diagnosis of infection with *Mycobacterium tuberculosis* has been the development of *in vitro* assays that measure the production of interferon (IFN)-γ in response to stimulation with *M. tuberculosis*-specific antigens (IFN-γ release assay (IGRA) tests). The QuantiFERON TB® Gold In-Tube (QFT-IT; Cellestis, Carnagie, Australia) measures IFN-γ responses by ELISA following incubation of whole blood with region of difference (RD)1 and TB7.7 (Rv2654) antigens. The T-SPOT.TB® test (Oxford Immunotech, Abingdon, UK) measures the number of IFN-γ responding cells by the enzyme-linked immunosorbent spot method following incubation of purified peripheral blood mononuclear cells (PBMCs) with RD1 antigens.

The IGRA tests have been extensively examined and current evidence suggests that both tests have a low false-positive rate [1, 2]. Compared with the tuberculin skin test (TST), the IGRAs are better correlated with risk factors for infection with *M. tuberculosis* and do not give false-positive responses in bacille Calmette–Guerin (BCG)-vaccinated individuals [3–5]. Additionally, in patients with active tuberculosis (TB) and especially in immunocompromised individuals the IGRAs are more sensitive than the TST [6–10]. However, there is a concern that the detection rate is still suboptimal and the tests perform with more indeterminate results, and a lower detection rate in patients with severe TB and immunocompromised patients [7, 9–12].

**For editorial comments see page 1428.**
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The detection rate of IGRA could potentially be enhanced with the addition of further *M. tuberculosis*-specific antigens [13], by improving the incubation step [14–16] or by measuring alternative or additional biomarkers for IFN-γ [16–20]. As part of the search for improved diagnostic measures, we have screened >30 potential biomarkers (as published previously [20] and observed in unpublished studies) and among those we have characterised IFN-γ inducible protein (IP)-10 [20] and monocyte chemotactic protein (MCP)-2 as potential *in vitro* biomarkers for infection with *M. tuberculosis*.

Both IP-10 and MCP-2 are proinflammatory chemokines [21] which are expressed in inflamed tissues by resident and infiltrated cells (primarily monocyte/macrophages) after paracrine stimulation from T-cells by IFNs and other proinflammatory cytokines, or through innate mechanisms upon contact with viral, bacterial and fungal agents [22–27]. IP-10 is involved in trafficking monocytes and activated T-helper cell type 1 cells to inflamed foci through interaction with the CXC chemokine receptor (CXCR)3 [28]. MCP-2 is chemotactic for and activates many different cell types, including granulocytes and mononuclear cells, through various chemokine receptors including chemokine (C-C motif) receptor (CCR)1, CCR2B and CCR5 [21, 27]. Serum and pleural fluid IP-10 levels have been evaluated as biomarkers for diagnosis, prognosis and monitoring of treatment efficacy in inflammatory and infectious diseases, including TB. MCP-2 is less explored, but comparable properties have been described [20, 21, 29–38]. Both IP-10 and MCP-2 can be induced in monocytes and macrophages present in PBMC or whole blood by antigen and mitogen stimulation [20, 25, 39].

The aims of the current study were to evaluate the potential of *in vitro* antigen-specific expression of IP-10 and MCP-2 for the diagnosis of *M. tuberculosis* infection and to evaluate whether IP-10 and MCP-2 could be used to improve the diagnosis of *M. tuberculosis* infection. The present study confirmed prior observations that IP-10 and MCP-2 could be used as a diagnostic biomarker in line with, or in addition to, IFN-γ.

## METHODS

### Study population

Material from 80 patients with active TB, 86 high school students and 38 high school staff were included. Patients with active TB based on positive culture and/or positive PCR were included from two centres in Europe: the Institute for Infectious Diseases of the University of Berne, Berne, Switzerland (n=46) and Copenhagen University Hospital, Copenhagen, Denmark (n=34). Clinical data was collected from patient files. Immunosuppression was classified according to LEE *et al.* [8]. Controls were included among students and staff at a high school in the greater Copenhagen area, Denmark (additional detail on the inclusion of controls is provided in the online supplementary material). The study protocol was approved by ethical committee of Copenhagen and Frederiksberg Municipal (KF 01-278477; Denmark) and by the ethical committee of Berne, Switzerland (KEK-BE 038/07).

### TST

Controls and TB patients were skin tested using 2TU RT-23 tuberculin (Statens Serum Institute, Copenhagen, Denmark); ⩾10 mm induration was considered positive.

### IFN-γ determination by Quantiferon

In total, 1 mL of whole blood was drawn into the three QFT-IT tubes (Cellestis; coated with saline (nil), *M. tuberculosis*-specific antigens or mitogen) and incubated for 18 h. After centrifugation, IFN-γ was measured in the supernatants by ELISA according to the manufacturer's instructions (Cellestis). IFN-γ results are shown in $pg \cdot mL^{-1}$ to facilitate comparisons with the other biomarkers. One International unit of IFN-γ corresponds to 50 $pg \cdot mL^{-1}$ (NIBSC, Potters Bar, UK).

### IP-10 and MCP-2 determination

On the same supernatants as above, IP-10 and MCP-2 were measured in duplicate by xMAP (Luminex Corporation, Austin, TX, USA) technology as previously described [20]. Samples were diluted 1:3 in assay diluent in order to optimise the expected IP-10 concentrations in the antigen tube to the range of the standard curve. Levels above the upper limit of quantification were assigned the upper limit (19,920 $pg \cdot mL^{-1}$ for IP-10 and 8,820 $pg \cdot mL^{-1}$ for MCP-2). The lower level of quantification was 20 $pg \cdot mL^{-1}$ for both biomarkers.

### Data analysis

Data were analysed using SAS 9.1.3 (SAS institute, Cary, NC, USA) and R (R Development Core Team, Vienna, Austria). Variables with normal distribution were described using mean ± SD and means were compared using a paired t-test. Variables that were not normally distributed were compared across and within groups using nonparametic tests (Kruskal–Wallis and Wilcoxon signed-rank test). Biomarker correlation was assessed using the Spearman's rank test. The antigen-dependent and the mitogen-induced biomarker production were measured by subtracting the concentration measured in the nil tube from the concentration measured in the antigen and mitogen tube, respectively.

The diagnostic performance of the antigen-dependent biomarker values *per se* was compared using the receiver operating characteristic curve (ROC) analysis and the area under the curve (AUC), as suggested by HANLEY and MCNEIL [40]. Cut-offs for antigen-dependent IP-10 and MCP-2 were estimated at various sensitivities and specificities and at the maximum Youden's index (YI), *i.e.* sensitivity + specificity - 1 [41].

The following criteria were defined for potential diagnostic IP-10 and MCP-2 tests: positive if the antigen-dependent biomarker production was above the selected cut-off; negative if the antigen-dependent biomarker production was below the selected cut-off and not indeterminate; and indeterminate if the antigen-dependent response was negative and the mitogen-induced response was <200 $pg \cdot mL^{-1}$ for the IP-10 test and 150 $pg \cdot mL^{-1}$ for the MCP-2 test. These cut-offs were arbitrarily chosen. Test concordance was assessed using κ statistics. The McNemar's test was applied when comparing marginal homogeneity, detection and false-positive rate. All tests were two-sided and p-values <0.05 were considered significant.

## RESULTS

### Study population

Plasma samples from 80 patients with active TB and 124 (86 high school students and 38 high school staff) controls were included (table 1). The TB patients were significantly older than the students and ethnically more diverse. In total, 50%

**TABLE 1** Demographic and clinical characteristics

| | Students | Staff | TB patients |
|---|---|---|---|
| **Subjects n** | 86 | 38 | 80 |
| **Age yrs** | 17.6±1.3 | 54.5±8.5 | 46.4±19.3 |
| **Males n** | 23 (27) | 13 (34) | 39(49) |
| **Ethnicity** | | | |
| Europe/North America | 86 (100) | 38 (100) | 40 (50) |
| Africa | 0 (0) | 0 (0) | 18 (23) |
| Asia | 0 (0) | 0 (0) | 21 (26) |
| Other (Tahiti) | 0 (0) | 0 (0) | 1 (1) |
| **Prior TB treatment/disease** | | | |
| No | 86 (100) | 38 (100) | 8 (10) |
| Yes | 0 (0) | 0 (0) | 2 (3) |
| Unknown | 0 (0) | 0 (0) | 70 (87) |
| **>2 months in TB endemic country** | | | |
| No | 86 (100) | 38 (100) | 6 (8) |
| Yes | 0 (0) | 0 (0) | 45 (68) |
| Unknown | 0 (0) | 0 (0) | 20 (25) |
| **TB type** | | | |
| Pulmonary | | | 42 (53) |
| Extrapulmonary | | | 32 (40) |
| Pulmonary and extrapulmonary | | | 6 (8) |
| **Co-morbidity** | | | |
| HIVpositive n/n tested | 0/0 (0) | 0/0 (0) | 10/56 (18) |
| Other immunosuppressive treatment or condition | 0 (0) | 0 (0) | 8 (10) |
| **TST >10 mm n/n done** | 0/80 (0) | 8/38 (21) | 9/12 (75) |
| **PCR** | | | |
| Positive | | | 60 (75) |
| Negative | | | 12 (15) |
| Not done | | | 8 (10) |
| **Microscopy** | | | |
| Positive | | | 44 (55) |
| Negative | | | 26 (33) |
| Not done | | | 10 (13) |
| **Culture** | | | |
| Positive | | | 68 (85) |
| Negative | | | 8 (10) |
| Not done | | | 4 (5) |

Data are presented as n (%) and mean±SD, unless otherwise stated. TB: tuberculosis; TST: tuberculin skin test.

were born in a high TB endemic country, and 62% had spent >2 months in a TB endemic country, 48% had extrapulmonary TB, 13% were HIV positive and 10% were considered relatively immunosuppressed (treated with >15 mg·day$^{-1}$ prednisolone (n=4), post-transplantation chemotherapy (n=1), haematologic malignancy (n=1) and chronic renal failure (n=2)). The students who were BCG unvaccinated were predominantly female, ethnic Danes with no history of TB exposure. All staff had been BCG vaccinated.

### *TST and QFT-IT results*

TST results were available for 15% (12 out of 80) of the TB patients, of whom 75% were positive. All controls underwent TST, 21% (eight out of 38) of staff but none of the 86 students were TST positive. Among the 80 patients, 65 (81%) were QFT-IT positive, and four (5%) were QFT-IT indeterminate, due to low mitogen response. All controls were QFT-IT negative and none were indeterminate.

**TABLE 2** Interferon (IFN)-γ, IFN-γ inducible protein (IP)-10 and monocyte chemotactic protein (MCP)-2 release after stimulation

| | Students | Staff | TB patients |
|---|---|---|---|
| **Subjects n** | 86 | 38 | 80 |
| **IFN-γ** | | | |
| Nil[#] | 3 (2–4) | 2 (2–3) | 9 (6–15)[§,ƒ] |
| Antigen-dependent[¶] | 0 (0–1) | 0 (0–2) | 215 (22–651)[§,ƒ] |
| Mitogen-induced[+] | 480 (465–514) | 472 (312–495) | 654 (139–1025) |
| **IP-10** | | | |
| Nil[#] | 54 (37–89) | 63 (36–141) | 197 (114–369)[§,ƒ] |
| Antigen-dependent[¶] | 36 (14–85) | 47 (11–283) | 2158 (582–5882)[§,ƒ] |
| Mitogen-induced[+] | 4409 (2755–7909) | 6591 (3081–8363) | 2012 (814–3646)[§,ƒ] |
| **MCP-2** | | | |
| Nil[#] | 5 (0–13) | 1 (0–12) | 6 (2–13)[##] |
| Antigen-dependent[¶] | 2 (2–9) | 4 (0–21) | 379 (44–1915)[§,ƒ] |
| Mitogen-induced[+] | 6001 (1511–8112) | 6877 (2157–8114) | 1195 (217–8097)[§,##] |

Data are presented as median concentration in pg·mL$^{-1}$ (interquartile range), unless otherwise stated. TB: tuberculosis. [#]: unstimulated; [¶]: *Mycobacterium tuberculosis* antigen-stimulated subtracted nil; [+]: phytohaemaglutinin stimulated subtracted nil; [§]: p<0.0001 *versus* students; [ƒ]: p<0.0001 *versus* staff; [##]: p<0.005 *versus* staff (Kruskal–Wallis test).

### *Biomarker levels*

TB patients produced significantly higher antigen-dependent levels of all biomarkers compared with the controls, whereas the mitogen-induced IP-10 and MCP-2 levels generally were lower (table 2). The TB patients showed significantly higher levels of IFN-γ and IP-10 in the nil samples compared with both students and staff. There were no significant differences between students and staff for any of the biomarkers (p>0.07). In figure 1 the individual measurements of antigen- and mitogen-induced responses are shown. TB patients produced IP-10 and MCP-2 in 16.8 (interquartile range 5.2–36.0) and 2.7 (0.6–6.2)-fold higher magnitude compared with IFN-γ (p<0.0001). There was no significant difference in any of the biomarker levels when comparing the relatively immunosuppressed and HIV infected with the TB patients without comorbidity.

### *Correlation*

A high correlation was observed in the antigen-dependent responses between IFN-γ and IP-10 (r=0.81) and between IFN-γ and MCP-2 (r=0.72; p<0.0001; Spearman). The mitogen-induced levels of IFN-γ correlated less with IP-10 (r=0.30) and MCP-2 (r=0.41), although it was still significant (p<0.0001; Spearman).

### *Diagnostic performance of IP-10 and MCP-2*

The diagnostic performance of the biomarkers was compared using the ROC curve analysis. Students and TB patients were

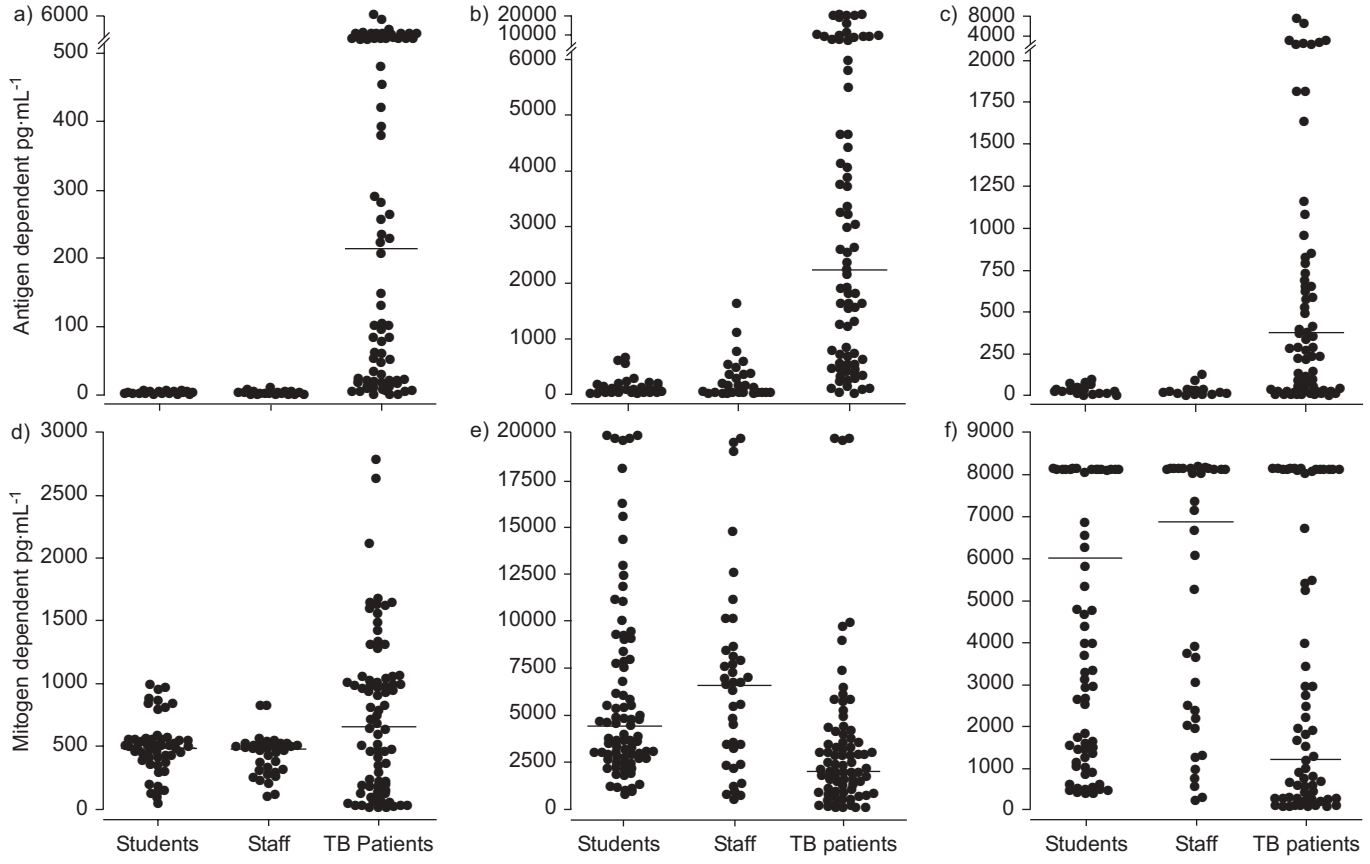


**FIGURE 1.** Whole blood was stimulated with *Mycobacterium tuberculosis*-specific antigens and with mitogen. The antigen-dependent (a–c) and mitogen-induced (d–f) interferon (IFN)-γ (a and d), IFN-γ inducible protein-10 (b and e) and monocyte chemotactic protein-2 (c and f) levels measured in blood samples from students, staff and in patients with active tuberculosis (TB). ●: an individual; ——— : median levels.

used as gold standard for noninfected and infected (fig. 2). There was no statistically significant difference between the AUC of IFN-γ, 0.98 compared with IP-10, 0.95 ($p=0.1$), but the AUC of MCP-2 (0.89) was significantly lower compared with IFN-γ and IP-10 ($p<0.04$).

### *Diagnostic tests based on IP-10 or MCP-2*

Like IFN-γ, IP-10 and MCP-2, responses to mitogen and antigen are inherently continuous; consequently, diagnostic test algorithms were used to convert them into positive, negative and indeterminate test results. The cut-offs for positive tests were estimated using ROC curve analysis on the antigen-dependent values (fig. 2). For IP-10, the maximum YI was achieved at the cut-off 237 pg·mL$^{-1}$ (detection rate 90%, false-positive rate 5%). Ideal false-positive rate (0%) was found using a higher cut-off (673 pg·mL$^{-1}$) with a lower detection rate (74%). It is likely that these two cut-offs represent the extremes in a range of potential cut-offs in the test. Since the aim of the current study was to explore the cut-off with high detection rate and low false-positive rate that was applicable in a clinical setting, a cut-off at 455 pg·mL$^{-1}$ (detection rate 80%, false-positive rate 3%) halfway between these two extremes was pragmatically selected for the evaluation of a potential IP-10 test. In addition, the performance of an IP-10 test based on the marginal IP-10 cut-offs (IP-10 test 237 pg·mL$^{-1}$ and IP-10 test 673 pg·mL$^{-1}$) was explored. For MCP-2 the cut-off was selected at maximum YI 95.7 pg·mL$^{-1}$ (detection rate 71%, false-positive rate 0%). There was no apparent alternative MCP-2 cut-off with an increasing detection rate, as the trade off in false-positive rate was too high (fig. 2). Maximum YI for IFN-γ was 4.0 pg·mL$^{-1}$ (detection rate 95%, false-positive rate 1%), whereas the cut-off recommended by the manufacturer is 17.5 pg·mL$^{-1}$ (detection rate 81%, false-positive rate 0%).

### *Test results and concordance*

The rate of positive, negative and indeterminate responders was calculated using the IP-10 and MCP-2 test algorithms and the results were compared with the QFT-IT test (table 3).

The rates of positive responders among TB patients were 81% (65 out of 80) for the QFT-IT, and 83% (66 out of 80) for the IP-10 test 455 pg·mL$^{-1}$. When excluding the indeterminate responders the detection rate increased to 86% (65 out of 76) and 89% (66 out of 74). The IP-10 test 455 pg·mL$^{-1}$ had a higher proportion of positive responders among all controls 7% (nine out of 124), with 3% (three out of 86) positive students and 16% (six out of 38) positive staff ($p<0.02$). For individual patient characteristics see online supplementary material. There was no association with immunosuppression and risk of false-negative test result for any of the tests ($p>0.33$). There was a very high agreement between the QFT-IT and the MCP-2 test (93%; κ 0.84); and the QFT-IT and the IP-10 test 455 pg·mL$^{-1}$ (89%; κ 0.77; $p<0.0001$;

**TABLE 3** Distribution of QuantiFERON TB® Gold In-Tube[#](QFT-IT) test, interferon-γ inducible protein (IP)-10 test 237 pg·mL$^{-1}$, IP-10 test 455 pg·mL$^{-1}$, IP-10 test 673 pg·mL$^{-1}$ and monocyte chemotactic protein (MCP)-2 test results among the students, staff and tuberculosis patients

| Test result | QFT-IT test | IP-10 test 237 pg·mL$^{-1}$ | IP-10 test 455 pg·mL$^{-1}$ | IP-10 test 673 pg·mL$^{-1}$ | MCP-2 test |
|---|---|---|---|---|---|
| **Patients** | | | | | |
| Positive | 65 (81) | 72 (90) | 66 (83) | 59 (74) | 57 (71)[¶] |
| Negative | 11 (14) | 4 (5) | 8 (10) | 15 (19) | 14 (18) |
| Indeterminate | 4 (5) | 4 (5) | 6 (8) | 6 (8) | 9 (11) |
| **Staff** | | | | | |
| Positive | 0 (0) | 10 (26)[+] | 6 (16)[§] | 3 (8) | 1 (3) |
| Negative | 38 (100) | 28 (74) | 32 (84) | 35 (92) | 37 (97) |
| Indeterminate | 0 (0) | 0 (0) | 0 (0) | 0 (0) | 0 (0) |
| **Students** | | | | | |
| Positive | 0 (0) | 4 (5) | 3 (3) | 0 (0) | 0 (0) |
| Negative | 86 (100) | 82 (95) | 83 (97) | 86 (100) | 86 (100) |
| Indeterminate | 0 (0) | 0 (0) | 0 (0) | 0 (0) | 0 (0) |

Data are presented as n (%). [#]: manufactured by Cellestis, Carnagie, Australia. [¶]: statistically different detection rate compared with QFT-IT ($p<0.04$); [+]: statistically different rate of positive responders (approximation of false-positive rate) compared with QFT-IT ($p<0.002$); [§]: statistically different rate of positive responders (approximation of false-positive rate) compared with QFT-IT ($p<0.02$).

see online supplementary material). The three tests agreed in 175 (86%) out of 204 of all samples examined (data not shown).

### *Increasing detection rate using marginal cut-offs and combining biomarkers*

The rate of positive responders was compared using the lower cut-offs for IP-10 and IFN-γ. The detection rate for the IP-10 test 237 pg·mL$^{-1}$ increased to 90% (72 out of 80), 95% (74 out of 75; excluding indeterminate) and to 26% (10 out of 38) among staff, but only to 5% (four out of 86) among students. With the IP-10 test 673 pg·mL$^{-1}$ the detection rate was lower 74% (59 out of 80), (80% (59 out of 74) excluding indeterminate), and the rate of positive responders was 8% (three out of 38) among staff and 0% (none out of 86) among controls (clinical data are provided in online supplementary material). Using the cut-off point for IFN-γ identified on the ROC curve as optimal (4 pg·mL$^{-1}$), the proportion of positive responders significantly increased from 81% (65 out of 80) to 95% (76 out of 80; 96% (76 out of 79) excluding indeterminate responders) and the positive rate among controls remained low at 2% (three out of 124).

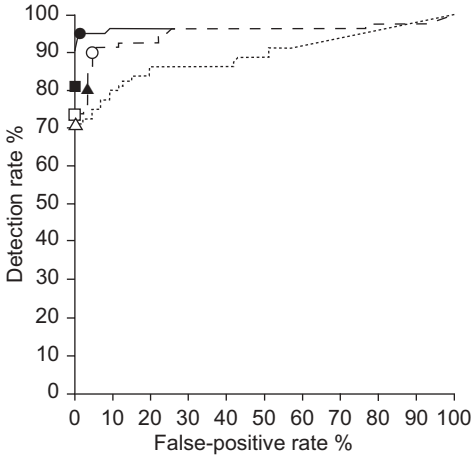


**FIGURE 2.** Whole blood was stimulated with *Mycobacterium tuberculosis*-specific antigens or saline. The diagnostic potential of interferon (IFN)-γ, IFN-γ inducible protein (IP)-10 and monocyte chemotactic protein (MCP)-2 was determined by receiver operating characteristic curve analysis using antigen-dependent values. Students were used as gold standard for noninfected; tuberculosis patients were used as gold standard for infected. ●: maximum Youden's index (YI) for IFN-γ (4 pg·mL$^{-1}$); △: maximum YI for MCP-2 (97 pg·mL$^{-1}$); ■: cut-off applied in the QuantiFERON In-Tube test (Cellestis, Carnagie, Australia; 17.5 pg·mL$^{-1}$); ○: maximum YI for IP-10 test, used as cut-off for the IP-10 test (237 pg·mL$^{-1}$); □: cut-off for the IP-10 test (673 pg·mL$^{-1}$); ▲: selected pragmatic cut-off for the IP-10 test (455 pg·mL$^{-1}$). ——: IFN-γ; – – – –: IP-10; ····: MCP-2.

Next, the authors evaluated if a combined biomarker approach could increase the detection rate without compromising specificity. A patient was classified as positive if at least one of two tests were positive and found that the combination of IP-10 test 455 pg·mL$^{-1}$ and QFT-IT increased the detection rate

**TABLE 4** Head-to-head comparison of test results among tuberculosis patients for the QuantiFERON TB® Gold In-Tube[#](QFT-IT) test and interferon-γ inducible protein (IP)-10 test 455 pg·mL$^{-1}$

| IP-10 test 455 pg·mL$^{-1}$ | QFT-IT | | | Total |
|---|---|---|---|---|
| | Negative | Positive | Indeterminate | |
| **Negative** | 5 | 2 | 1 | 8 |
| **Positive** | 6 | 59 | 1 | 66 |
| **Indeterminate** | 0 | 4 | 2 | 6 |
| **Total** | 11 | 65 | 4 | 80 |

Agreement 83%; κ=0.44. [#]: manufactured by Cellestis, Carnagie, Australia.

**TABLE 5** Head-to-head comparison of test results among tuberculosis patients for the QuantiFERON TB® Gold In-Tube# (QFT-IT) test and interferon-γ inducible protein (IP)-10 test 673 pg·mL$^{-1}$

| IP-10 test 673 pg·mL$^{-1}$ | QFT-IT | | | Total |
|---|---|---|---|---|
| | Negative | Positive | Indeterminate | |
| Negative | 7 | 7 | 1 | 15 |
| Positive | 4 | 54 | 1 | 59 |
| Indeterminate | 0 | 4 | 2 | 6 |
| Total | 11 | 65 | 4 | 80 |

Agreement 89%; κ=0.43. #: manufactured by Cellestis, Carnagie, Australia.

to 90% (72 out of 80; $p<0.009$; 92% (72 out of 78) excluding indeterminate responders; table 4). The positivity rate among controls remained low 7% (nine out of 124). Interestingly, when combining the IP-10 test 673 pg·mL$^{-1}$ with the QFT-IT, the detection rate increased significantly to 88% (70 out of 80; $p<0.03$; 90% when excluding indeterminate responders; table 5), with a positive rate among the controls of 2% (three out of 124). Combining MCP-2 with the QFT-IT or the IP-10 test did not increase the detection rate significantly ($p=0.08$ and $p=0.3$, respectively; table 6).

## DISCUSSION

The current study evaluated the potential of using antigen-specific IP-10 and MCP-2 expression for *in vitro* diagnosis of *M. tuberculosis* infection. After stimulation of whole blood with *M. tuberculosis*-specific antigens, IP-10 and MCP-2 were expressed by infected individuals and in significantly higher amounts than IFN-γ. IP-10 and MCP-2 tests were established, which performed with excellent concordance with the QFT-IT. By combining the results of IP-10 and IFN-γ, it was possible to increase the detection rates among TB patients 81–90% without a significant increase in positive responders among the students.

There is a demand for new diagnostic tests for TB with improved detection rates and which are simpler to perform, *i.e.* with a bedside readout similar to that of the lateral flow technique as used in the rapid tests for malaria and HIV [42, 43]. Several new promising technologies for active TB have emerged [44] but since the introduction of the RD1 antigen-based IGRAs in 2000, no major breakthroughs have been made to improve the diagnostic tools for latent infection [8, 42, 43].

**TABLE 6** Head-to-head comparison of test results among TB patients QuantiFERON TB® Gold In-Tube# (QFT-IT) test with the monocyte chemotactic protein (MCP)-2 test

| MCP-2 test | QFT-IT | | | Total |
|---|---|---|---|---|
| | Negative | Positive | Indeterminate | |
| Negative | 8 | 6 | 0 | 14 |
| Positive | 3 | 54 | 0 | 57 |
| Indeterminate | 0 | 5 | 4 | 9 |
| Total | 11 | 65 | 4 | 80 |

Agreement 83%; κ=0.55. #: manufactured by Cellestis, Carnagie, Australia.

Alternative biomarkers expressed in higher amounts than IFN-γ could improve the detection rate of the *in vitro* tests for *M. tuberculosis* infection, but so far no alternatives have shown to have similar or superior potential compared with IFN-γ. RD1-antigen stimulated expression of interleukin (IL)-2, IL-4, IL-10, macrophage inflammatory protein-1α, MCP-1, monokine induced by IFN-γ, IL-8 and the cell activation surface-marker CD40L have been examined, but responses were either inconsistent, difficult to measure, or lower compared with IFN-γ [16–20].

IGRA tests are primarily tools developed to diagnose latent *M. tuberculosis* infection [1], but due to the lack of a gold standard for latent *M. tuberculosis* infection, active TB is often used as a model to evaluate test performance [45]. QFT-IT is a robust IGRA, and several large studies have documented a high detection rate in TB patients without comorbidity and very low false-positive rate in healthy individuals from low endemic regions [1]. The performance of the QFT-IT in the present study was comparable with other studies on similar material [1, 45, 46]. The IP-10 test 455 pg·mL$^{-1}$ and the MCP-2 test performed comparably to the QFT-IT and the three tests agreed in 89% of all samples examined.

The range of potential IP-10 cut-offs with high detection rate and low false-positive rate led the current authors to evaluate test performance at different cut-offs. In the range 237–673 pg·mL$^{-1}$, detection rates were obtained ranging 74–90% among TB patients, with positivity rates in students 0–6% and staff 8–26%. Interestingly, the majority of controls that had a positive reaction with the IP-10 test were staff and aged >50 yrs. It is well known that there is an increasing risk of *M. tuberculosis* exposure with age and the positive responses in these individuals could possibly reflect prior *M. tuberculosis* exposure. However, the current study was not designed to evaluate latent TB infection and studies in persons with known exposure to patients with active TB are needed to test this hypothesis.

Applying the 4 pg·mL$^{-1}$ (0.08 IU·mL$^{-1}$) cut-off for IFN-γ in the present study population resulted in an increase in detection rate to 96% with only minor increase in false-positive rate to 2%. The calculations support the increasing number of papers discussing whether the QFT-IT cut-off could be lowered to increase QFT-IT performance [8, 47, 48], it is however not sufficiently explored if reduced cut-off for the QFT-IT will compromise the very low false-positive rate seen in most studies [1] and whether these very low IFN-γ concentrations can be reliably reproduced with serial or repeated testing [9, 49].

The most striking finding of the study was that the detection rate was increased using a combined biomarker approach. Depending on the IP-10 cut-off detection rate increased from 81 to 88% or 90%, with only minimal influence on the rate of false-positive controls. This combined biomarker approach would be an easy way to improve the already existing tests.

Interestingly the same adjunct effects were not seen on QFT-IT combination with the MCP-2 test.

The present authors have demonstrated the performance of IP-10 and MCP-2 in an exploratory study using ''the sickest of the sick and the wellest of the well'' [50]. The limitation of this approach is the risk of over-optimistic cut-offs and over-estimated test accuracy, and the present study design does not allow the determination of the sensitivity, specificity, positive and negative predictive values or likelihood ratio of the new tests. These qualities need to be determined in another population.

In the present study the xMAP assay was used, an assay which has not been optimised for this specific purpose. Further optimisation of the readout platform could result in improved test performance and the optimal cut-off for an IP-10 test needs to be confirmed in clinically relevant challenge studies [50]. Such studies should focus on the performance of the new tests in patients with other inflammatory or infectious disorders, and in latently infected individuals. Furthermore neither the IGRA nor the IP-10 or MCP-2 test, at this stage, solves the issue of active TB *versus* latent TB infection, *e.g.* diagnosing active TB in patients with clinical suspicion of TB within a population with a high prevalence of latent TB infection.

An algorithm for the IP-10 and MCP-2 tests was used that was similar to the algorithm developed for IFN-γ. The optimal algorithm for an IP-10 or MCP-2 based test, however, may differ from that of IFN-γ.

In conclusion, there is a need for added sensitivity when screening high-risk populations, *e.g.* immunocompromised patients and for the diagnosis of patients with active tuberculosis. The present data suggests that monocyte chemotactic protein-2 and, especially, interferon-γ inducible protein-10 could be promising new biomarkers, as they are produced antigen specifically in high amounts by tuberculosis patients and not by controls. Most interestingly, the present authors have demonstrated that by simply combining the measurement of interferon-γ inducible protein-10 and interferon-γ test accuracy improved significantly.

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