



Prospective evaluation of host biomarkers other than interferon gamma in QuantiFERON Plus supernatants as candidates for the diagnosis of tuberculosis in symptomatic individuals

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SUMMARY

Background: There is an urgent need for new tools for the diagnosis of TB. We evaluated the usefulness recently described host biomarkers in supernatants from the newest generation of the QuantiFERON test (QuantiFERON Plus) as tools for the diagnosis of active TB.

Methods: We recruited individuals presenting at primary health care clinics in Cape Town, South Africa with symptoms requiring investigation for TB disease, prior to the establishment of a clinical diagnosis. Participants were later classified as TB or other respiratory diseases (ORD) based on the results of clinical and laboratory tests. Using a multiplex platform, we evaluated the concentrations of 37 host biomarkers in QuantiFERON Plus supernatants from study participants as tools for the diagnosis of TB.

Results: Out of 120 study participants, 35(29.2%) were diagnosed with active TB, 69(57.5%) with ORD whereas 16(13.3%) were excluded. 14(11.6%) of the study participants were HIV infected. Although individual host markers showed potential as diagnostic candidates, the main finding of the study was the identification of a six-marker biosignature in unstimulated supernatants (Apo-AIII, CXCL1, CXCL9, CCL8, CCL-1, CD56) which diagnosed TB with sensitivity and specificity of 73.9%(95% CI: 51.6–87.8) and 87.6%(95% CI: 77.2–94.5), respectively, after leave-one-out cross validation. Combinations between TB-antigen specific biomarkers also showed potential (sensitivity of 77.3% and specificity of 69.2%, respectively), with multiple biomarkers being significantly different between TB patients, QuantiFERON Plus Positive and QuantiFERON Plus negative individuals with ORD, regardless of HIV status.

Conclusions: Biomarkers detected in QuantiFERON Plus supernatants may contribute to adjunctive diagnosis of TB.

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Introduction

An estimated 10.4 million people were reported to have fallen ill with tuberculosis (TB), and another 1.6 million died of the disease in 2017.¹ The currently existing diagnostic tests have several widely publicised limitations^{2,3} including poor sensitivity (smear microscopy),⁴ long turnaround times and high costs (culture).⁵ The

GeneXpert MTB/RIF test (Cepheid, Sunnyvale, CA, USA), is relatively rapid and detects resistance to rifampicin, but is also expensive, with low specificity in recurrent TB.⁶ These tests are not very suitable for people with difficulty in providing good quality sputum samples such as children and those with extrapulmonary TB.⁷ Immunological approaches have been shown to be potentially useful in these forms of TB, and may be easily converted into point-of-care screening tests.^{5–7}

The tuberculin skin test (TST) and interferon gamma (IFN- γ) release assays (IGRAs) remain the only approved immunodiagnostic tests for the management of TB. As these tests cannot distinguish

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between latently infected individuals and those with active TB,^{8,9} they are not recommended for diagnosis of active TB in high burden settings, due to the high prevalence of LTBI.¹⁰ Many investigators have evaluated alternative antigens other than those used in IGRAs (ESAT-6 and CFP-10)^{11–13} and alternative host biomarkers other than IFN- γ ^{8,12} as biomarkers for active TB. Investigations done in QuantiFERON TB Gold (QFT) In Tube culture supernatants in particular, identified promising biosignatures for the diagnosis of active pulmonary TB in both adults^{14,3, 16} and children.^{15,17} The QFT In Tube test which contained ESAT-6, CFP-10 and TB7.7 peptides has been replaced by the recently introduced QFT Plus, which only contains CFP-10 and ESAT-6 peptides, but includes a second TB antigen tube.¹⁸ The first TB antigen tube (TB1) contains long peptides that reportedly elicit CD4 T cell immune responses and the TB2 tube contains both short and long peptides which elicit CD4 and CD8 T cell immune responses.¹⁹

According to the manufacturer (Qiagen, Germany), the QFT Plus reportedly has high sensitivity (>95%) and boasts the highest specificity of any test for the diagnosis of *Mycobacterium tuberculosis* (*M. tb*) infection.¹⁸ Given that host immunological biomarkers detected in QFT In Tube supernatants were shown to have potential as diagnostic biomarkers for active TB in several studies, reviewed in,²⁰ it is not known whether the findings from these studies can be replicated in supernatants from the QFT Plus system. Given the reportedly higher sensitivity and specificity of the QFT Plus,¹⁸ the use of alternative biomarkers detectable in QFT Plus supernatants may be useful in the diagnosis of active TB, as shown in a recent report with IP-10.²¹ Biosignatures based on such antigen-specific responses may enhance the specificity of host biomarker-based tests. In the present study, we evaluated the utility of multiple host immunological biomarkers that have previously been shown to possess diagnostic potential for active TB in QFT Plus supernatants. We also included biomarkers that showed potential in recent studies done on serum or plasma specimens^{27, 28} and other recently identified host biomarkers, as diagnostic candidates for active TB in QFT Plus supernatants from individuals suspected of having TB disease in a high TB endemic setting.

Materials and methods

We prospectively recruited individuals presenting at primary health care clinics in Cape Town, South Africa with symptoms requiring investigation for TB disease, prior to the diagnosis of TB or other diseases as part of a larger ongoing multisite project (ScreenTB; www.screen-tb.eu). Participants were enrolled between November 2016 and October 2017 (Fig. 1). Participants were eligible for inclusion in the study if they self-reported at the clinic with symptoms or signs suggestive of TB, namely coughing for ≥ 2 weeks in addition to at least one other symptom or sign including fever, weight loss, haemoptysis, malaise, night sweats, chest pain or loss of appetite. Other eligibility criteria included age between 18 and 70 years, and willingness to give written informed consent and undergo HIV testing. Participants were excluded if they had not been living in the study community for up to 3 months, had no permanent address, if they were pregnant or breast feeding, had HB<9 g/l, were on TB treatment or had received anti-TB treatment in the previous 90 days, if they were HIV positive and on isoniazid prophylaxis, and if they had been treated with quinolone or aminoglycoside antibiotics in the previous 60 days. The study was approved by the Health Research Ethics Committee of the University of Stellenbosch.

Collection of specimens and diagnostic tests

Whole blood was collected by venepuncture, directly into QFT Plus tubes (1 mL to each of the nil, TB1, TB2 and mitogen tubes) as

recommended by the manufacturer (Qiagen, Germany). Tubes were transported at ambient conditions to the laboratory after which they were incubated for 18 to 20 h at 37 °C, in a 5% CO₂ incubator. Tubes were then centrifuged at 3000 x g for 15 min as recommended by the manufacturer and supernatants harvested, aliquoted and frozen at –80 °C until use. Sputum samples collected from all study participants were tested for *M. tb* using the GeneXpert, followed by culture using the MGIT method (BD Biosciences). Specimens which showed growth of microorganisms were further examined for acid fast bacilli using the Ziehl–Neelsen method, followed by and Capilia TB testing (Tauns, Japan) to confirm the isolation of organisms of the *M.tb* complex before being classified as positive cultures.

Reference standard for classification of study participants

Participants were classified as either definite TB, probable TB or other respiratory diseases (ORD) using a composite reference standard combination of clinical, laboratory and radiological findings as previously described.^{3,22} As in previous studies,^{3,23,24} individuals with ORD had a variety of other diagnoses which included upper and lower respiratory tract infections and acute exacerbations of chronic obstructive pulmonary diseases or asthma. However, more extensive investigations including bacterial and viral cultures were not done to confirm all the ORDs as it is not standard practice in the primary health care clinics where study participants were recruited.

QuantiFERON-TB gold plus ELISA

IFN- γ concentrations in QFT Plus supernatants were determined using standard QFT Plus ELISA kits. All experiments were done and data analysed and interpreted using the analysis software provided by the manufacture (Qiagen). ELISA results were only used for determination of the *M. tb* infection status of study participants and not for patient management.

Luminex multiplex immunoassay

The concentrations of 37 host biomarkers that were identified as TB diagnostic candidates after literature searches^{14,23,38} were evaluated in the nil, TB1 and TB2 antigen stimulated culture supernatants from all study participants. These included apolipoprotein (Apo) A-1, Apo-CIII, complement factor H (CFH), IFN- α 2, macrophage inflammatory protein 1 alpha (MIP-1 α /CCL3), interleukin (IL)-4, IL-6, in reagent kits purchased from Merck Millipore, Billerica, MA, USA, and neural cell adhesion molecule-1 (NCAM-1/CD56), transforming growth factor (TGF)- α , IFN- γ , monokine induced by IFN- γ (CXCL9/MIG), p-selectin, epidermal growth factor (EGF), tumour necrosis factor superfamily member 14 (TNFSF14/LIGHT), monocyte chemotactic protein (MCP-1/CCL2), MCP-2(CCL8), MIP-1 β (CCL4), vascular endothelial growth factor-A (VEGF-A), von Willebrand factor-cleaving protease (ADAMTS13), CD40 ligand (CD40L), interferon inducible T-cell alpha chemoattractant (CXCL11/ITAC-1), IFN- γ inducible protein-10 (IP-10/CXCL10), tumour necrosis factor (TNF)- α , Fas, IL-1 β , granulocyte monocyte colony stimulating factor (GM-CSF), IL-8(CXCL8), IL-3, IL-15, IL-13, IL-2, IL-33, IL-1 α , IL-10, IL-1 receptor agonist (IL-1ra), and IL-22, which were in reagent kits purchased from R&D Systems Inc. (Biotechne, Minneapolis, MN, USA). All experiments were performed in a blinded manner, on the Bio Plex platform (Bio-Rad Laboratories, Hercules, USA) with the Bio-Plex Manager Software (version 6.1) used for bead acquisition and analysis of median fluorescent intensity. The values of analytes in the respective quality control reagents evaluated on all plates, were within their expected reference ranges.

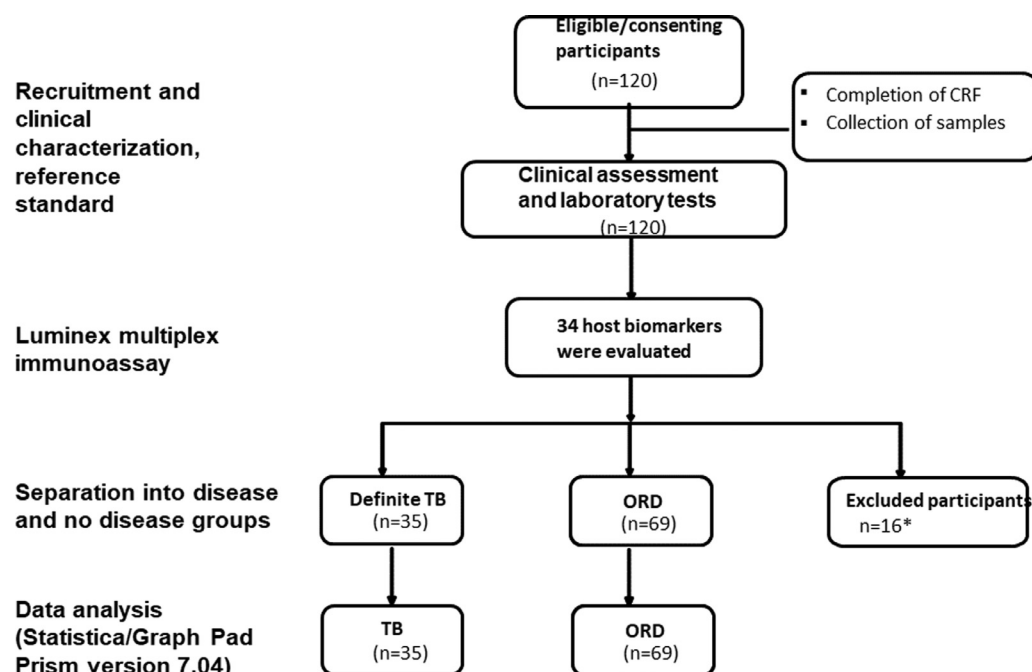


Fig. 1. STARD flow diagram showing the study design and classification of participants. *These study participants were excluded either because they had culture contamination in addition to having negative sputum smear results ($n=8$) or because of loss to follow-up during TB workup ($n=8$). TB =pulmonary tuberculosis, ORD=other respiratory diseases; that is, patients in whom TB disease was ruled out.

Statistical analysis

The Mann-Whitney U test was used for assessing differences in the concentrations of individual host biomarkers between any two groups e.g., TB vs. ORD. The diagnostic accuracy of individual markers was evaluated by receiver operator characteristics (ROC) curve analysis. Cut-off values and associated sensitivity and specificity were determined using the Youden's Index. The General discriminant analysis (GDA) procedure was used to assess the predictive abilities of combinations between different host biomarkers. Prediction accuracy was ascertained by leave-one-out cross validation. P -values ≤ 0.05 were considered significant. Data were analysed using Graph Pad Prism version 7.04 (San Diego, CA, USA) and Statistica (TIBCO Software Inc., CA, USA).

Results

A total of 120 study participants were enrolled into the current study, 14(11.6%) of whom were HIV infected. 16 (13.3%) of the study participants were excluded for reasons explained in Fig. 1. The mean age of the remaining 104 participants was 40.1 ± 12.6 years and 40(38.5%) were males. Using the manufacturer's recommended cut-off value (≥ 0.35 IU/mL), 87(72.5%) of all study participants were QFT Plus positive. We intended to employ our pre-established composite reference standard comprising of clinical information, laboratory (smear, GeneXpert and culture) and imaging findings to classify study participants as definite TB, probable TB, possible TB or ORD as reported in previous studies.^{3,14} However, all participants that were finally diagnosed with TB in the present study ($n=35$; 29.2%) were culture and GeneXpert positive, with 24 (68.6%) being smear positive (Table 1).

Utility of individual biomarkers in the diagnosis of TB

We evaluated the differences in the concentrations of biomarkers detected in culture supernatants after stimulation with the TB1 and TB2 antigens, and the nil (unstimulated) responses sep-

Table 1

Clinical and demographic characteristics of study participants with a tuberculosis or other respiratory disease diagnosis. Data for the 16 excluded study participants not shown.

	All	TB disease	ORD
No. of patients	104	35 (29.1%)	69 (70.8%)
Female's n (%)	64 (53.3%)	10 (28.5%)	46 (66.6%)
Age mean $\pm$ SD	40.1 ± 12.6	34.8 ± 12.1	41.9 ± 12.6
HIV positive n (%)	9 (8.7%)	7 (20.0%)	2 (3%)
QFT plus Positive n (%)	87 (72.5%)	27 (77.1)	49 (71%)
QFT plus Negative n (%)	32 (26.7%)	8 (22.8)	20 (28.9)
Indeterminate n (%)	1 (1%)	1 (2.9%)	0 (0%)

We intended to classify all study participants as definite TB, Probable TB, possible TB or ORD using the pre-established diagnostic algorithm described in (26). However, all individuals classified as TB in this study were culture and GeneXpert positive (definite TB). Participants classified as ORD were culture negative, smear negative and had no TB suggestive chest X rays. Furthermore, none of these participants was treated for TB by the TB control program. Abbreviations: TB = Tuberculosis, ORD = other respiratory disease, SD = standard deviation, HIV = Human immunodeficiency virus, QFT = QuantiFERON TB Gold Plus.

arately to assess the contribution of unstimulated biomarkers in the diagnosis of active TB as previously reported.^{11,14} TB1 and TB2 responses were corrected for background by subtraction of the unstimulated values prior to analysis. We first evaluated the correlation between IFN- γ values determined using the QFT Plus ELISA (IU/mL) and what was detected using the Luminex platform (pg/mL). As observed in previous studies,^{14,24} there was a significant positive correlation between IFN- γ values detected by ELISA and Luminex in both the nil ($r=0.2164$), and the TB1($r=0.4278$, and TB2 ($r=0.526$) stimulated values, at $p < 0.05$).

Host markers detected in unstimulated (nil) supernatants: when the concentrations of host markers detected in the QFT Plus nil supernatants from TB patients were compared to responses obtained in individuals with other respiratory diseases (ORD) with the Mann Whitney U test irrespective of HIV infection status, significant differences ($P \leq 0.05$) were observed for the concentrations

Table 2

Median concentrations of host markers (Inter-quartile ranges in parenthesis) detected in unstimulated (nil) culture supernatants and accuracies in the diagnosis of TB disease ($n=35$) and other respiratory diseases ($n=69$). Only biomarkers that showed significant differences ($p \leq 0.05$) between groups with the Mann Whitney U test are shown. AUC= Area under the ROC curve, CI= Confidence interval. Cut-off values were selected based on the Youden's Index. All concentrations are in pg/mL with the exception of Apo-A1 and Apo-CIII (ng/mL).

Marker	ORD ($n=69$) (IQR)	TB disease ($n=35$) (IQR)	P value	AUC (95%CI)	Cut-off value	Sensitivity (95% CI)	Specificity (95% CI)
ITAC-1	310 (238–450)	561 (381–753)	0.0001	0.77 (0.67–0.80)	>381.70	78 (56–92)	62 (48–73)
IL-3	35 (24–43)	44 (38–48)	0.0008	0.75 (0.63–0.86)	>40.85	76 (53–92)	65 (52–76)
I-309	11 (8–13)	18 (10–26)	0.0001	0.73 (0.62–0.84)	>11.09	71 (54–85)	55(43–67)
MIG	890 (772–1095)	1133 (873–1645)	0.0014	0.73 (0.60–0.85)	>940.30	70 (47–87)	57 (44–69)
Apo- CIII	281,738 (169,471–838,212)	122,386 (80,337–295,193)	0.0013	0.69 (0.59–0.80)	<203,602	71 (54–85)	65 (53–76)
IL-1 α	43 (23–67)	62 (47–78)	0.0066	0.69 (0.57–0.81)	>50.87	74 (52–90)	62 (49–73)
IL-33	20 (3–45)	41 (29–55)	0.0065	0.69 (0.58–0.81)	>28.20	78 (59–92)	58 (45–71)
Apo-A1	710,225 (394,468–1,525,800)	346,446 (190,034–653,295)	0.0032	0.68 (0.57–0.59)	<536,691	73 (54–87)	65 (53–76)
ADAMTS13	1,418,300 (1,061,200–1,762,700)	71,289 (71,289–1,496,500)	0.0137	0.67 (0.54–0.81)	<203,602	67 (47–86)	64 (57–80)
IL-2	1180 (563–1552)	1555 (1275–1707)	0.0145	0.67 (0.55–0.76)	>1363	70 (47–87)	65 (50–75)
NCAM-1	126,811 (19,041–154,833)	100,864 (75,770–132,990)	0.0047	0.67 (0.56–0.78)	<124,132	72 (53–86)	54 (41–66)
GM-CSF	37 (23–56)	62 (25–94)	0.0134	0.65 (0.53–0.77)	>40.81	71 (54–85)	55 (43–65)
IL-22	28 (15–41)	36 (31–44)	0.0389	0.65 (0.53–0.77)	>29.12	90 (69–99)	57 (44–66)
TGF- α	23 (18–34)	31 (19–51)	0.0213	0.64 (0.53–0.76)	>28.32	64 (45–80)	65 (53–76)

Table 3

Median concentrations of host markers (Inter-quartile ranges in parenthesis) detected in TB1 antigen stimulated culture supernatants and accuracies in the diagnosis of TB disease. Only biomarkers that showed significant differences ($p \leq 0.05$) or trends ($0.05 < p \leq 0.1$) between groups with the Mann Whitney U test are shown. AUC= Area under the ROC curve, CI= Confidence interval. Cut-off values were selected based on the Youden's Index. All concentrations are in pg/mL.

Markers	ORD($n=69$) (IQR)	TB($n=35$) (IQR)	P value	AUC (95% CI)	Cut-off value	Sensitivity (95% CI)	Specificity (95% CI)
P-Selection	0.197 (–0.3806–0.6405)	–0.4173 (–1.447–0.4049)	0.0277	0.66 (0.52–0.80)	0.04329	68 (45–86)	60 (47–72)
MIP-1 α	–0.1339 (–0.2646–0.3281)	0.2562 (–0.537–0.04445)	0.0816	0.64 (0.53–0.76)	–0.1554	70 (51–84)	55 (43–67)
IL-2	–0.137 (–0.2132–0.7377)	–0.088795 (–0.0341–(–0.1149)	0.0491	0.64 (0.52–0.74)	0.4564	68 (45–86)	60 (44–69)
LIGHT	–0.0934 (–0.7485–0.7281)	–0.4049 (–0.792–0.1191)	0.0794	0.63 (0.50–0.73)	–0.2076	71 (48–89)	52 (40–65)
IL-13	–0.0892 (0.2827.518)	–0.2463 (–0.518–0.0051)	0.0484	0.62 (0.51–0.57)	–0.128	70 (0.51–0.84)	54 (41–66)
GM-CSF	0.2213 (–0.3758–0.2015)	–0.2611 (–0.7483–0.402)	0.05834	0.62 (0.51–0.73)	–0.02804	66 (48–81)	62 (50–74)
IL-10	0.2727 (–0.2287–0.7325)	0.00814 (–0.4544–0.2281)	0.0548	0.62 (0.51–0.73)	0.2201	73 (55–88)	52 (40–64)

Values shown were background corrected by subtraction of the unstimulated from the antigen-stimulated concentration for each study participant. A negative value before a number indicates that the unstimulated concentrations of the biomarker were higher than the TB1 antigen-stimulated values.

of 14 of the 37 markers evaluated. The median concentrations of ITAC-1, IL-2, IL-3, I-309, MIG, ADAMTS13, IL-1 α , GM-CSF, IL-33, IL-22, and TGF- α were significantly higher in the TB patients whereas, the concentrations of Apo-A1, Apo-CIII and NCAM-1 were significantly higher in the ORD group (Table 2). When the diagnostic accuracy of these proteins were assessed using ROC curve analysis, the areas under the ROC curves (AUCs) were ≥ 0.65 for 13 of these analytes, with ITAC-1, IL-3, I-309, and MIG being the most accurate individual markers (AUCs > 0.70). When only the HIV uninfected individuals were considered, only the same 14 host markers showed significant differences between the two groups.

Host markers detected in TB1 antigen-specific supernatants: when the TB1 antigen-specific (TB1 antigen-minus-nil) host markers, were compared between the TB and ORD groups, the median levels of seven host markers were significantly different between the two groups. The concentrations of MIP-1 α and IL-2 were significantly higher in TB patients whereas those of p-selectin, IL-13, IL-10, LIGHT, and GM-CSF were higher in ORD group (Table 3). These seven host markers diagnosed TB with AUC ≥ 0.60 after ROC curve analysis, with MIP-1 α , p-selectin, and IL-2 being the most accurate individual markers.

Host markers detected in TB2 antigen-specific supernatants: when the TB2 antigen-specific concentrations of the host markers (TB2 antigen-minus-nil) were compared between the TB and ORD groups, significant differences were obtained for the median levels of seven markers. The levels of TGF- α were higher in the TB group whereas those of p-selectin, EGF, IL-10, IL-13, MIP-1 α and GM-CSF were higher in the ORD group (Table 4). EGF and p-selectin were the most accurate TB2 antigen-specific individual biomarkers with AUCs of 0.66 (95%CI, 0.54–0.81) and 0.65 (95% CI, 0.52–0.78), respectively, for the diagnosis of active TB.

Utility of combinations between different biomarkers in the diagnosis of TB

When the data obtained from the *unstimulated culture supernatants* were fitted to GDA models regardless of HIV infection status of study participants, a six-marker biosignature comprising of Apo-A1, ITAC-1, I-309, MIG, MCP-2 and NCAM-1 diagnosed TB with an AUC of 0.91 (95% CI, 0.88–0.97), corresponding to a sensitivity of 73.9% (95% CI, 51.6–89.8%) and specificity of 87.6% (95% CI, 77.2–94.5%). After leave-one-out cross-validation, the sensitivity of the biosignature was 73.9% (95% CI, 51.6–89.8%) and specificity was 86.2% (95% CI, 75.3–93.5%). The positive and negative predictive values (PPV and NPV) of the biosignature (after leave-one-out cross validation) were 68.0% (95% CI, 51.5–81.0%) and 90.5% (95% CI, 82.6–91.0%) respectively (Fig. 2(A)). When only the HIV uninfected participants were considered, a four-marker unstimulated biosignature comprising of Apo-CIII, I-309, MIG and NCAM similarly diagnosed TB disease with an AUC of 0.91 (95% CI, 0.85–0.98), corresponding to sensitivity of 82.4% (95% CI, 56.6–96.2%) and specificity of 87.3% (95% CI, 76.5–94.4%). The sensitivity and specificity of the biosignature were 76.5% (95% CI, 50.1–93.2%) and 85.7% (95% CI, 74.6–93.3%) respectively after leave-one-out cross validation, with PPV and NPV of 59.1% (95% CI, 42.8–73.4%) and 93.1% (95% CI, 85.0–96.8%), respectively (Table 5 and, Fig. 2(B)).

When the *TB1 antigen-specific* marker data were similarly fitted into GDA models, regardless of HIV infection status, a four-marker signature comprising of Apo-CIII, I-309, MIP-1 α and TNF- α diagnosed TB disease with an AUC of 0.72 (95% CI, 0.61–0.88), with sensitivity and specificity of 62.9% (95% CI, 44.9–78.2%) and 73.3% (95% CI, 66.7–87.3%) respectively. After leave-one-out cross validation, the sensitivity and specificity of the biosignature were 54.3% (95% CI, 36.7–71.2%) and 72.5% (95% CI, 60.4–82.5%) respectively

Table 4

Median concentrations of host markers (Inter-quartile ranges in parenthesis) detected in TB2 antigen stimulated culture supernatants and accuracies in the diagnosis of TB disease. Only biomarkers that showed significant differences ($p \leq 0.05$) or trends ($0.05 < p \leq 0.1$) between groups with the Mann Whitney *U* test are shown. AUC= Area under the ROC curve, CI= Confidence interval. Cut-off values were selected based on the Youden's Index. All concentrations are in pg/mL.

Marker	ORD(<i>n</i> = 69) (IQR)	TB(<i>n</i> = 35) (IQR)	<i>P</i> value	ACU (95% CI)	Cut-off value	Sensitivity (95% CI)	Specificity (95% CI)
P-Selectin	0.1928 (−0.402–0.7307)	−0.2961 (−1.35–0.2293)	0.0161	0.66 (0.54–0.81)	0.03092	76 (53–92)	60 (47–72)
EGF	0.6551 (−0.4189–0.5737)	0.2845 (0.7855–0.0088)	0.0333	0.65 (0.52–0.78)	−0.01726	77 (55–92)	62 (49–73)
GM-CSF	0.1559 (−0.3593–0.6323)	−0.1459 (−0.6546–0.1884)	0.0327	0.63 (0.51–0.75)	−0.1396	66 (48–81)	65 (53–75)
IL-10	0.1892 (−0.5080–0.6323)	−0.5043 (0.8695–0.5049)	0.0384	0.63 (0.51–0.74)	0.09878	73 (54–88)	61 (48–72)
IL-13	−0.2060 (−0.4324–0.7489)	−0.3809 (−0.7489–0.2386)	0.0860	0.61 (0.41–0.72)	−0.2277	64 (40–85)	54 (41–66)
MIP-1 α	−0.1482 (−0.389–0.3574)	−0.294 (0.6531–0.9609)	0.0914	0.60 (0.49–0.72)	−0.1986	70 (48–82)	55 (43–67)
TGF- α	0.086 (−0.8910–0.3405)	0.152 (−0.5266–0.9270)	0.02068	0.56 (0.45–0.70)	−0.08228	60 (38–63)	51 (38–63)

Values shown were background corrected by subtraction of the unstimulated from the antigen-stimulated concentration for each study participant. A negative value before a number indicates that the unstimulated concentrations of the biomarker were higher than the TB2 antigen-stimulated values.

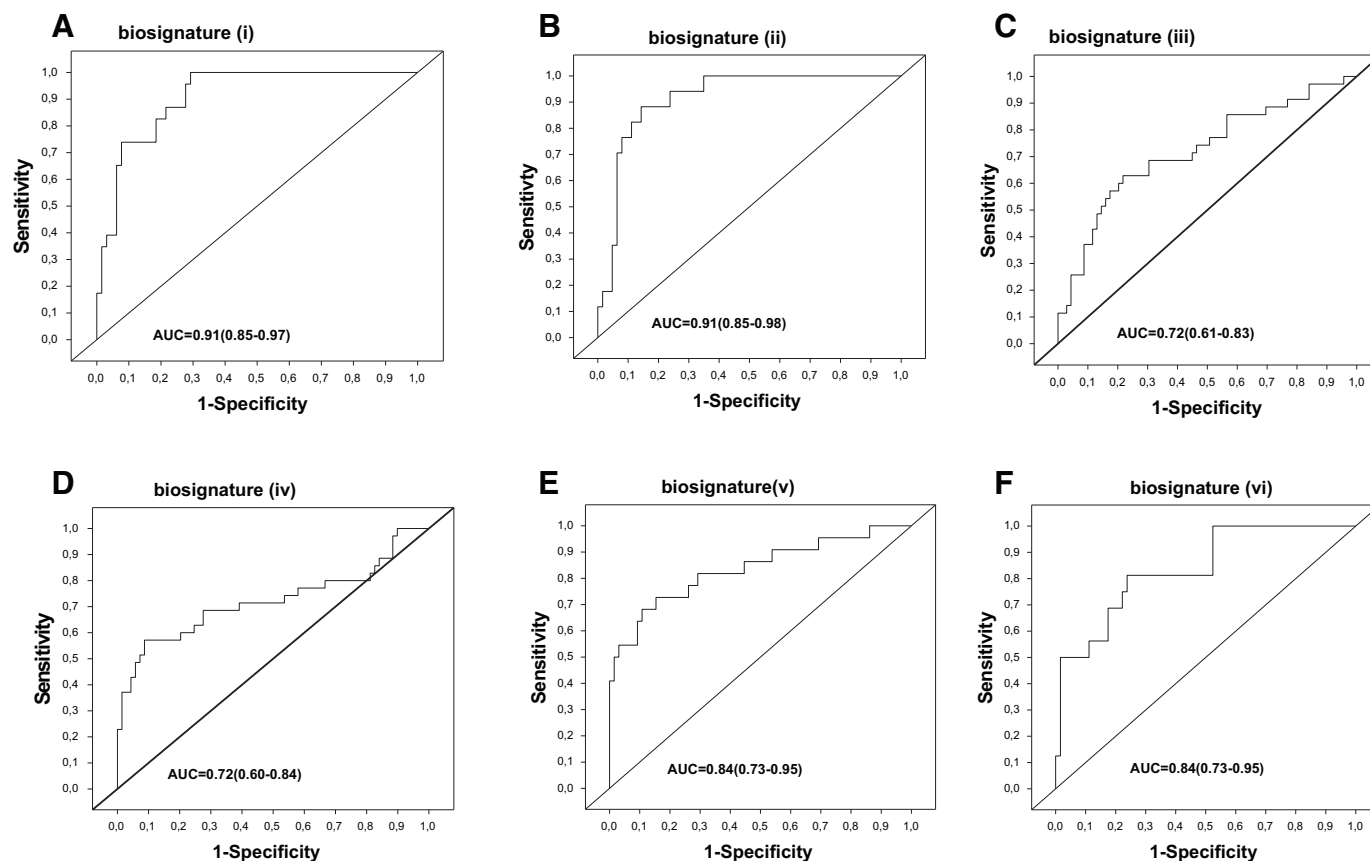


Fig. 2. Usefulness of combinations of host markers detected in QFT Plus supernatants in diagnosis of TB disease. ROC curves showing the accuracies of the different biosignatures described in the text and Table 5. A, ROC curve showing the accuracy of biosignature (i): combinations between unstimulated host markers regardless of HIV infection. B, ROC curve showing the accuracy of biosignature (ii): combinations between unstimulated markers in HIV uninfected individuals. C, ROC curve showing the accuracy of biosignature (iii): combinations between TB1 antigen-specific host markers regardless of HIV infection. D, ROC curve showing the accuracy of biosignature (iv): combinations between TB2 antigen-specific host markers regardless of HIV infection. E, ROC curve showing the accuracy of biosignature (v): combinations between TB1&TB2 antigen-specific host markers regardless of HIV infection. F, ROC curve showing the accuracy of biosignature (vi): combinations between TB1&TB2 antigen-specific host markers in HIV uninfected individuals.

(Table 5 and Fig. 2(C)). When TB-2 antigen-specific data were fitted into GDA models, again regardless of HIV infection status, a four-marker biosignature comprising of IL-6, MIP-1 α , GM-CSF and TGF- α diagnosed TB disease with an AUC of 0.72 (95% CI, 0.60–0.84), corresponding to a sensitivity 60.0% (95% CI, 42.0–76.1%) and specificity of 79.1% (95% CI, 68.3–88.4%). After leave-one-out cross validation, the sensitivity of the biosignature was 57.1% (95% CI, 65.1–86.1%) and the specificity was 71.6% (95% CI, 39.4–73.7%) (Table 5 and Fig. 2(D)).

When we evaluated combinations between TB1 and TB2 antigen-specific biomarkers, again, regardless of HIV infection status, a four-marker biosignature comprising of TNF- α , LIGHT, MIG, and p-selectin diagnosed TB disease with a sensitivity of 77.3% (95% CI,

54.6–92.2%) and specificity of 72.3% (95% CI, 59.8–82.7%). After leave-one-out cross validation, the sensitivity and specificity of the biosignature were 77.3% (95% CI, 54.5–92.2%) and 69.2% (95% CI, 56.6–80.1%), respectively (Table 5, Fig. 2(E) and (F)).

Differential expression of host biomarkers in TB patients and Quantiferon Plus positive vs. Quantiferon Plus negative individuals with ORD

We evaluated the ability of the different host markers in discriminating between TB patients, QFT Plus positive, and QFT Plus negative patients with ORD.

Table 5

Summary of biosignatures identified in the current study. AUC=Area under the ROC curve, Spec= Specificity, Sens= Sensitivity, PPV= Positive predictive values, NPV= Negative predictive value.

Biosignature	AUC (95% CI)	Classification matrix		Leave-one-out cross validation			
		Sens (%)	Spec (%)	Sens (%)	Spec (%)	PPV (%)	NPV (%)
<i>Biosignature (i): Markers detected in unstimulated (nil) supernatants, regardless of HIV infection</i>							
APO-CII+CXCL1/ITAC-1+CXCL9/MIG+CCL8/MCP-2+I-309+NCAM-1/CD56	0.91 (0.88–0.97)	73.9 (17/23)	87.3 (57/65)	73.9 (17/23)	86.2 (56/65)	68.0 (51.2–80.9)	90.5 (82.6–95.0)
<i>Biosignature (ii): Markers detected in unstimulated (nil) supernatants, only in HIV uninfected participants</i>							
CXCL9/MIG+CCL1/I-309+CCL8/MCP-2+NCAM-1/CD56	0.91 (0.85–0.98)	82 (14/17)	87 (55/63)	76.5 (13/17)	85.7 (54/63)	59.1 (42.1–73.6)	93.1 (85.1–97.0)
<i>Biosignature (iii): TB1 antigen-specific biomarkers (TB1 Ag-minus nil), regardless of HIV infection status</i>							
CFH+CCL1/I-309+CCL4/MIP-1 α +TNF- α	0.72 (0.61–0.88)	62.9 (22/35)	78.3 (54/69)	54.3 (19/35)	72.5 (50/69)	60.0 (46.7–71.1)	80.6 (72.6–86.7)
<i>Biosignature (iv): TB2 antigen-specific biomarkers (TB2 Ag-minus nil), regardless of HIV infection status</i>							
IL-6+CCL4/MIP-1 α +GM-CSF+TGF- α	0.72 (0.60–0.84)	60.0 (21/35)	79.7 (55/69)	57.1 (15/35)	76.7 (53/69)	60.0 (46.6–72.0)	79.7 (72.0–85.7)
<i>Biosignature (v): Combinations between TB1 and TB2 antigen-specific biomarkers, regardless of HIV infection status</i>							
TNF α +LIGHT+CXCL9/MIG+P-Selectin	0.84 (0.73–0.95)	77.3 (17/22)	72.3 (47/65)	77.3 (17/22)	69.2 (45/65)	48.6 (37.5–59.8)	81.1–95.4
<i>Biosignature (vi): Combinations between TB1 and TB2 antigen-specific biomarkers, only in HIV uninfected patients</i>							
ADAMTS13+CCL1/I-309+CCL4/MIP-1 α +p-selectin	0.84 (0.73–0.95)	62.5 (10/16)	82.5 (52/63)	56 (9/16)	76 (48/63)	37.5 (24.4–52.7)	87.3 (79.5–92.4)

Using the Mann Whitney *U* test, 16 of the proteins detected in unstimulated supernatants including I-309, ITAC-1, IL-3, IL-33, MIG and Apo-CIII (see Supplementary Table 1) were significantly different between individuals with TB disease and QFT Plus positive individuals with ORD. Apo-A1, MIG, Apo-CIII, ITAC-1 and IL-1 α levels were significantly different between TB patients and QFT Plus negative individuals with ORD whereas, none of the 37 biomarkers detected in unstimulated supernatants showed differences between QFT Plus positive and QFT Plus negative individuals with ORD (Supplementary Table 1).

When the TB1 antigen specific (TB1Ag-nil) host markers were compared between the three groups, 14 proteins including IL-13, MIP-1 α , IL-3, GM-CSF, IL-1 α and IL-10 (see Supplementary Table 2) were significantly different between the TB patients and QFT plus positive individuals with ORD. Surprisingly, only IP-10 and p-selectin were significantly different between patients with active TB and QFT Plus negative individuals with ORD, whereas IFN- γ , IL-13, IL-2, MCP-2, and ITAC-1 antigen-specific levels were significantly different between QFT Plus positive and QFT Plus negative individuals with ORD (Supplementary Table 2).

When the TB2 antigen-specific host markers (TB2 Ag-nil) were compared between groups, nine proteins including IL-6, IL-13, GM-CSF, IL-10, IL-2, and MIP-1 α showed significant differences between TB patients and QFT Plus positive individuals with ORD. When the TB patients were compared to QFT Plus negative individuals with ORD, only IFN- γ and p-selectin levels were significantly different, whereas TB2 antigen-specific IFN- γ , MIP-1 α , IL-22, IL-13, and GM-CSF were significantly different between QFT Plus positive and QFT Plus negative individuals with ORD (Supplementary Table 3).

Discussion

In the current study, we evaluated the usefulness of host markers that showed potential in previous studies including antigen-stimulated (mainly QFT In tube) culture supernatants as well as serum and plasma specimens, as diagnostic candidates for TB disease. As the QFT Plus test is reportedly more sensitive and specific for *M. tb* infection than the QFT In Tube; the previous generation of the test,¹⁸ we hypothesized that host biomarkers

that previously showed potential, especially in QFT In tube supernatants will perform with improved accuracy. Many individual host biomarkers that are detectable in either unstimulated, TB1 or TB2 antigen-stimulated supernatants including ITAC-1, IL-3, I-309, MIG, Apo-A1, p-selectin, MIP-1 α , EGF, GM-CSF, IL-2 and IL-10 showed potential in the diagnosis of TB disease in this study. However, as observed in previous studies (14, 29, 36), combinations between different biomarkers were more accurate than individual analytes, with combinations of analytes detected in the unstimulated supernatants being the most accurate in the diagnosis of TB disease.

Out of the 14 host markers that showed potential in diagnosing TB disease in unstimulated supernatants, four, namely, ITAC-1, IL-3, I-309 and MIG were amongst the most promising analytes identified in plasma in a previous study.²⁵ Other host biomarkers which showed potential including EGF, TGF- α , IL-2, IL-33, Apo-A1, ADAMTS13, GM-CSF and IP-10 also showed potential in previous studies.^{3,15,28–33} Our findings from unstimulated culture supernatants are therefore in agreement with these previous studies, and indicate that these proteins may be strong candidate biomarkers for the diagnosis of active TB.

It is widely known that CD4 T cells play an important role in the immune response against *M. tb* infection through production of IFN- γ and other host markers. However, in addition to CD4 T cells, evidence from previous studies have shown that CD8 T cells also play an important role during *M. tb* infection through production of cytokines.^{18,34,35} The addition of CD8+ T-cell stimulating peptides into one of the QFT Plus tubes is hypothesised to have increased the sensitivity for diagnosing *M. tb* infection as well as active TB.^{18,36} In this study however, the most promising host markers for the diagnosis of active TB were detected in unstimulated supernatants and not supernatants from the antigen containing tubes. Antigen-specific biomarkers that showed potential after stimulation with the TB2 antigens included IL-13, TGF- α , MIP-1 α , EGF, GM-CSF, IL-10 and p-selectin.

IL-13 is a cytokine that is secreted by many cell types including CD4 T cells, natural killer cells, eosinophils amongst others and is mostly associated with T helper 2 immunity.³⁷ MIP-1 α is a chemoattractant that is mainly produced by macrophages after stimulation with bacterial endotoxins. IL-10 is an anti-inflammatory cytokine which inhibits the synthesis of

pro-inflammatory cytokines include IFN- γ , TNF- α and IL-2 which are mainly produced by T cells and macrophages.³⁸ GM-CSF is a glycoprotein which functions as a cytokine and is produced by various immune cells including T cells, natural killer cells and macrophages. Its role is to stimulate stem cells which produce granulocytes and monocytes³⁹ whereas p-selectin is a cell adhesion molecule which is found on the surface of the endothelial cells.⁴⁰ EGF and TGF- α are growth factors. EGF stimulates cell growth and differentiation amongst other functions and enhances the growth of extracellular and intracellular *M. tb* at the site of infection.⁴¹ TGF- α plays an important role as an EGF receptor ligand, which activates the signalling pathway for proliferation, activation and development of the cell. Upregulation of these growth factors in QFT supernatants from TB patients was demonstrated in previous studies.³ We only observed the findings for the growth factors in the TB2 and not TB1 culture supernatants. It is not clear whether CD4 or CD8 T cells were the main producers of these proteins. If only CD8 T Cells, then it does not explain the observation of these proteins in previous QFT In Tube based studies (14, 27) and requires further investigation. QFT Plus is meant for the diagnosis of *M. tb* infection. We observed differences in the expression of some of the biomarkers evaluated between patients with active TB, QFT Plus positive patients with ORD and QFT Plus negative patients with ORD as shown in the supplementary files. However, we did not perform extensive analysis for these differences as this was not the main focus of the current study. Our goal was to evaluate the performance of biomarkers in diagnosing active TB, regardless of whether individuals presenting with symptoms were latently infected or not, as this study was conducted in a high burden setting. As observed in previous studies, combinations between multiple host biomarkers performed better in the diagnosis of TB disease,^{26,42,43} with a different optimal, and smaller biosignature obtained if only HIV uninfected participants were considered. We were not sufficiently powered to perform a detailed analysis of the influence of HIV infection on the biomarkers as only 14 of the study participants, and only two out of the 69 individuals in whom TB was ruled out (3% of the ORD group) and 7 out of the 35 TB patients (20%) were infected with HIV. Combinations between antigen-specific biomarkers were inferior to the performance of biomarkers obtained in unstimulated supernatants and for optimal diagnosis using antigen-specific biomarkers, our data is in favour of combinations between both the TB1 and TB2 antigen-specific markers.

Given the recent findings that biomarkers detectable in serum, plasma, urine and saliva samples may be useful, and even performed better than the accuracy obtained in QFT Plus supernatants in the current study,^{12,23,29,40} the utility of QFT Plus-based host biomarkers might be limited. The use of such antigen-specific host markers may be important in specific niche groups in the difficult-to-diagnose TB including childhood and extrapulmonary TB.⁴⁴ More research is needed to ascertain the position of such biomarkers in the TB diagnostic landscape.

The strengths of the current study include the prospective, phase III design and the fact that it was conducted in an area with one of the highest TB burdens in the world, with a high prevalence of LTBI¹ and our findings may be relevant regarding field applicability of the. However, our sample size was relatively small, owing to costs. Future studies should evaluate the most promising analytes identified in the present study in samples obtained from multiple sites. The severity of HIV infection in any infected participants should be determined, and used to assess the possible influence of HIV on the accuracy of the biomarkers. Such studies may include children and patients with extrapulmonary TB.

In conclusion, our findings indicate that some of the host markers identified as TB diagnostic candidates in previous studies show potential as diagnostic candidates for TB disease, when measured

in QFT Plus supernatants. Biosignatures obtained from unstimulated culture supernatants were more promising than antigen-stimulated biomarkers, indicating that ex vivo blood, serum or plasma based assays may be preferable to antigen-stimulated assays. Our findings require validation in other, larger studies.

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Potential conflicts of interest

GW and NC are listed as inventors on a South African Patent (Application No: ZA2009/05156).

Supplementary materials

Supplementary material associated with this article can be found, in the online version, at doi:[10.1016/j.jinf.2019.07.007](#).

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