**Introduction**

The diagnosis of tuberculosis (TB) disease in resource-poor settings remains challenging. Several independent studies have reported on the limitations of current techniques in diagnosing TB.1-4 There is a lack of simple field-friendly diagnostic tools and markers of immune activation and modulation of cytokine networks during intracellular infections might provide opportunities to develop appropriate tools.5-11

The Interferon gamma (IFN-γ) release assays (IGRAs) with high specificity and accuracy in the diagnosis of *Mycobacterium tuberculosis* (*Mtb*) infection have been widely employed in the immune-based diagnosis of *Mtb* infection and have some advantages over the tuberculin skin test.12 However, IGRAs are mainly useful in low incidence settings and for research advances in high burden areas as their major disadvantage is the inability to differentiate between active and latent TB.12,13 The discovery of secreted biomarkers similar to the gene expression signatures that were recently identified and that differentiate between these two infection states and which can be further developed into a rapid point of care test would be a major boost in TB diagnosis.14

Recently, there has been an upsurge in the alternative use of novel *Mtb* antigens and host markers besides IFN-γ in *Mtb-*specific antigen stimulated whole blood culture assay for exploring the diagnosis of TB.15 We have previously measured many of these host markers including tumour necrosis factor (TNF-α), interferon-inducible protein (IP-10), epidermal growth factor (EGF), macrophage inflammatory protein (MIP)-1β, vascular endothelial growth factor (VEGF) and soluble CD40 ligand (sCD40L) after stimulation with novel *Mtb* infection phase-dependent antigens (including TB vaccine candidate antigens, dormancy (DosR) regulon encoded antigens, TB reactivation antigens, TB resuscitation promoting factors (rpfs) and other stress response-associated antigens) in whole blood culture supernatants and some of these antigens look promising in TB disease diagnosis.16,17 However, in these studies, long term (7 day) whole blood assays were employed, which is not ideal for diagnostic purposes. In a follow up to these studies, we evaluated the potential of some of these promising antigens to elicit a host response in a short term (overnight) whole blood assay compared to the long term (7 day) whole blood assay.18 This study also evaluated the accuracy of some of these previously reported novel candidate antigens but in a larger study employing a short term (overnight), more field-friendly whole blood assay.

**Materials and methods**

**Study participants**

All the participants presumed of having pulmonary TB who participated in this study were recruited as part of the EDCTP funded African European Tuberculosis Consortium (AE-TBC) study that was conducted across six different African countries (www.ae-tbc.eu). Participants included in the present study were recruited from field sites serving Stellenbosch University, South Africa; Makerere University, Uganda; Medical Research Council Unit, The Gambia; and Karonga Prevention Study, Malawi. Participants presented with symptoms suggestive of pulmonary TB disease such as persistent cough for more than 2 weeks and one of the following: fever, recent loss of weight, night sweats, haemolysis, chest pain or loss of appetite. Participants were eligible for the study if they were 18 years or older, willing to give written informed consent, including for HIV testing using a rapid test (Abott, Germany) and sample storage. The exclusion criteria included severe anaemia (HB<10g/l), pregnancy, other known diseases such as diabetes mellitus, current anti-TB treatment, anti-TB treatment in the last 90 days, use of quinolone or aminoglycoside antibiotics in the past 60 days, and not been resident in the study area for more than 3 months. A case report form was completed for each participant before the collection of blood, saliva and other intended samples including urine and sputum as required for the main study. Culture of sputum samples was done using the MGIT method (BD Biosciences) and confirmation of isolated *Mtb* complex in all positive cultures was carried out by an *Mtb* complex specific PCR or standard biochemical methods, dependent on the facilities available at the study site.4 Additionally, 3 ml of blood was collected from the participants for the performance of QFT-IT assay, which was carried out according to the manufacturer’s instruction as previously described.19 The Human Ethics Research Committee of the University of Stellenbosch gave approval for the study (N10/08/274).

**Reference standard for classification of study participants**

Prior to the commencement of recruitment of study participants, harmonized case definitions were established and used for the classification of study participants (presumed TB cases) at all study sites. Participants were classified as having definite TB, probable TB, questionable TB disease status or non TB, using a combination of clinical, radiological, and laboratory findings.45 The non TB cases were cases had a range of other diagnoses, including upper and lower respiratory tract infections (viral and bacterial infections, although attempts to identify organisms by bacterial or viral cultures were not made), and acute exacerbations of chronic obstructive pulmonary disease or asthma. No participant in the non TB group underwent TB treatment during the 6 month follow up of the study. In assessing the diagnostic accuracy of the markers investigated in the present study, all the definite and probable TB cases were classified as “TB”, and then compared to the non TB cases, whereas questionables were excluded (Figure 1).

**Whole blood culture assay (WBA)**

At enrolment, 10ml of heparinised blood was collected from all participants and transported at ambient conditions within two hours of collection to the laboratory where the WBA was performed. The antigens that were used came from two sources namely: Leiden University Medical Center (LUMC), The Netherlands, and the Statens Serum Institut (SSI), Denmark. ESAT-6 and CFP-10 are two separate antigens, but were measured together as a fusion protein (ESAT-6/CFP-10) in this study. ESAT-6/CFP-10 and RV0081 were selected for the current study because of the promising accuracy shown by host markers elicited by these antigens in our previous studies16-18 whereas Rv1284 and Rv2034 were selected because of the promise already shown by the antigens as TB diagnostic and vaccine candidates in previous studies.20,21 Prior to their usage the four lyophilised antigens were reconstituted in sterile 1X PBS. The reconstituted antigens were then diluted in sterile 1x PBS, mixed with undiluted whole blood from each study participant at a final concentration of 10µg/ml, and incubated overnight (20-24hours) in 24-well tissue culture plates (Corning Corstar, Sigma) as previously described.18 Sterile 1x PBS (Lonza, Cat #: 17-517Q) was used as the negative control.

**Luminex multiplex immunoassay**

This prospective study included 322 TB and non TB cases and was evaluated using a Luminex multiplex cytokine platform which is based on simultaneous detection and cytometric quantification of different cytokines in a sample. The concentrations of 42 host markers including interleukin (IL)-1β, IL-1Rα, IL-2, IL-4, IL-5, IL-6, IL-7, IL-8, IL-9, IL-10, IL-12, IL-13, IL-15, IL-17, eotaxin, fibroblast growth factor (FGF) basic, granulocyte colony stimulating factor (G-CSF), granulocyte monocyte colony stimulating factor (GM-CSF), IFN-γ, interferon inducible protein (IP)-10, monocyte chemotactic protein (MCP)-1, macrophage inflammatory protein (MIP)-1α, platelet derived growth factor BB (PDGF-BB), MIP-1β, RANTES, TNF-α, vascular endothelial growth factor (VEGF), eotaxin-2, BCA-1, 6Ckine, SCF, TRAIL, ENA, ferritin, fibrinogen, procalcitonin, serum amyloid protein A (SAA), tissue plasminogen activator, serum amyloid protein P (SAP), CRP, haptoglobin and α-2 macroglobulin, were evaluated in WBA supernatants of all the study participants. This was done using Milliplex kits (Merck Millipore, St. Charles, Missouri, USA) and Bio-Plex kits (Bio Rad Laboratories, Hercules, CA, USA) on the Bio-Plex™ platform according to the manufacturer’s instructions. Standard curves were generated from the serial dilutions that were made from the assay controls supplied and matched against the cytokine concentration for quantification. The concentrations of all the analytes in the quality control reagents were found to be within the ranges as expected. The Bio-Plex manager version 6.1 was used for bead acquisition and analysis of median fluorescence intensity.

**Statistical analysis**

Statistical differences in analyte levels were evaluated by the Mann Whitney U test for non-parametric data analysis. The diagnostic accuracies of individual antigen-specific or ustimulated responses for TB disease were ascertained by receiver operator characteristics (ROC) curve analysis. Cut-off levels for estimation of sensitivity and specificity were selected based on the Youden’s Index. The predictive abilities of combinations of unstimulated and antigen-specific host markers for TB disease and non TB were investigated by performing best subsets general discriminant analysis (GDA). Data were randomly partitioned into a 70% training data set, which was used for model building and 30% test set, which was used to verify the accuracy of the different models. The leave-one-out cross validation approach was used to test the prediction accuracy of biosignatures after data was stratified according to HIV status, due to the relatively limited number of HIV infected individuals. Data were analyzed using GraphPad prism, version 5.00 for Windows (Graphpad Software, San Diego California, USA) and Statistica (Statsoft, Ohio, USA).

**Results**

**Study participants**

A total of 322 participants were enrolled into this study, 106 (33%) of who were cultures positive TB cases (Figure 1). Of the 322 study participants, 168 (52%) were males and 24 (23%) of the 106 TB cases were HIV co-infected. The demographic and clinical information of the participants are shown in table 1.

**Potential of host markers produced by unstimulated supernatants in discriminating between individuals with TB and non TB disease**

When the analyte levels detected in the unstimulated control supernatants in TB patients were compared to the levels obtained in the non TB group (50% of this group were QFT-IT positive), the unstimulated levels of 14 out of the 42 host markers evaluated showed significant differences. The concentrations of these markers including CRP, Ferritin, IP-10, IL-6, IL-7, IL-9, IL-13, IFN-γ, VEGF, Haptoglobin, SAP, PCT and SAA were significantly higher in the TB group (Table 2). When the diagnostic potentials of these unstimulated host markers were evaluated by ROC curve analysis, four analytes including CRP, IP-10, Ferritin and SAA had an area under the ROC curve (AUC) of ≥ 0.85, ≥ 0.74, ≥ 0.79 and ≥ 0.77 respectively, in unstimulated samples. At their optimal unstimulated cut-off values, SAA had a sensitivity and specificity of 81% and 72%, ferritin 70% for both sensitivity and specificity, IP-10 had 77% sensitivity and 71% specificity for ascertaining TB disease. The best performance characteristic was with unstimulated CRP with a sensitivity and specificity of 80% (Table 2, Figure 3). The high AUC recorded for some of these markers support their diagnostic potential.

**Utility of host markers detected in overnight antigen-stimulated culture supernatants in the diagnosis of TB disease**

The unstimulated control levels for the different host markers were subtracted from the antigen-stimulated responses for each study participant before the analysis of the data. In response to *Mtb*-specific antigenic stimulation by ESAT-6/CFP-10, median concentrations of IP-10, IFN-γ, IL-1Rα, tPA and TRAIL were significantly higher in the TB group (p<0.05) (Table 2, Figure 2). Following stimulation with Rv2034, IL-2, IL-17 and FGF basic levels were significantly higher in TB cases whereas ferritin was higher in non TB. Rv1284 elicited the production of significantly high levels of IL-2 in the non TB cases, whereas only tPA responses were significantly different between the TB and non TB cases after stimulation with Rv0081 (Table 2). When the diagnostic accuracy of individual antigen-specific host markers were investigated by ROC curve analysis, the AUCs for ESAT-6/CFP-10 stimulated IP-10 and IFN-γ were ≥ 0.64 respectively. Antigen-specific level of IP-10 had the best sensitivity of 60% and specificity of 65%. The AUC’s of Rv1284-specific and Rv2034-specific markers performed poorly in general. Only Rv2034-specific level of IL-2 attained 0.60 (Table 2, Figure 3).

**Ability of cytokine responses to discriminate between LTBI and uninfected controls**

When the concentration of host markers detected in QFT-IT positive non TB cases (LTBI) were compared to the levels obtained in the QFT-IT negative non TB cases (uninfected controls), the unstimulated levels of IL-1β, IL-1Rα, IL-6, IL-10, IL-12, MIP-1α, TNF-α and were significantly higher in the uninfected controls. Only unstimulated levels of eotaxin were significantly higher in LTBI subjects. When the host markers elicited after stimulation with the different antigens were compared between the two groups, most of the discriminatory markers were found in ESAT-6/CFP-10 stimulated supernatants. ESAT-6/CFP-10 -specific levels of IL-1Rα, IL-2, IL-4, IL-5, IL-13, IL-15, FGF basic, GM-CSF, IFN-γ, IP-10, MCP-1, MIP-1α and Eotaxin-2 were significantly higher in the LTBI group. Similarly, Rv2034-specific levels of IL-8, IL-15, MCP-1 and MIP-1α, and Rv1284-specific levels of G-CSF, MCP-1 and PDGF-BB were significantly higher in the LTBI. Stimulation with Rv0081 failed to elicit any response. When the diagnostic accuracies of the markers detected in the culture supernatants were evaluated by ROC curve analysis, only ESAT-6/CFP-10-specific levels of IP-10, IFN-γ, GM-CSF, IL-2 and IL-13 discriminated between the two groups with AUC ≥ 0.70. Out of these five markers, ESAT-6/CFP-10-specific level of IP-10 had the best sensitivity and specificity of 75% and 72% respectively. Although ESAT-6/CFP-10-specific IL-5 and eotaxin-2, Rv2034-specific MCP-1, and Rv1284-specific PDGF-BB all discriminated between the two groups with sensitivities >80%, the specificities of all these markers were poor, ranging between 38-50% (Table 3).

**Abilities of combinations of analytes in the general discriminant analysis models in discriminating between TB and non TB.**

To evaluate the predictive abilities of combinations of analytes for TB and no TB disease data obtained from all study participants were analysed by general discriminant analysis (GDA), regardless of the HIV infection status of the study participants. The unstimulated and antigen-specific responses of each host marker were treated as separate variables, in order to evaluate the contribution of both classes of markers in predictive models. We randomly partitioned all the data from the measurement of the different markers into a 70% training data set for model building, and 30% for a test set for the verification of the models. A combination of six markers IP-10 Ag-Nil, IFN-γ Ag-Nil, IP-10Nil**,** Ferritin Nil, SAA Nil, and CRP Nil accurately predicted 77% TB cases and 84% of the non TB cases in the training set, regardless of HIV infection status. In the test set, the six-marker biosignature accurately predicted 83% of the TB cases and 78% of the non TB cases (Table 4).

To investigate the influence of HIV infection on the accuracy of the biosignatures, data was stratified according to HIV status, and the GDA procedure repeated. In the HIV uninfected group the six-marker biosignature (IP-10 Ag-Nil, IFN-γ Ag-Nil, IP-10Nil**,** Ferritin Nil, SAA Nil, and CRP Nil) diagnosed TB disease with a sensitivity of 83% and specificity of 90% in the training data set, and a sensitivity of 88% and specificity of 82% in the test dataset. However, the combination of these analytes performed less well in the HIV infected patients as only 64% of the TB cases and 80% of the non TB cases were correctly classified in the resubstitution classification matrix. After leave-one-out cross validation, the biomarker combination only resulted in the correct prediction of 52% of the TB cases and 76% non TB (Table 4). The frequency of the different analytes in the top 20 models for discriminating between TB disease and non TB in all study participants is shown in figure 4.

**Discussion**

The development of a new, relatively rapid, and accurate test, that does not rely on sputum, which can be difficult to obtain in some patient groups, and which does not reflect the site of infection in extrapulmonary TB, would be a major advance in the TB diagnostic field. The measurement of a small number of analytes that differentiates active TB from LTBI in the blood in a short-term overnight assay, might fulfil this need.19 Test results would be available within 48 hours, rather than after several weeks as is the case with sputum culture. In this study we investigated the potential accuracy of host markers detected in supernatants, after stimulation of whole blood with *Mtb* infection phase-dependent antigens, in an overnight culture assay. We have shown that multiple biomarkers detected in the antigen-stimulated and unstimulated supernatants can contribute to a diagnostic signature with the ability to discriminate between active TB and non TB. A biosignature of six analytes showed promising results especially in HIV uninfected individuals. We previously reported on the potential of host markers produced after stimulation of blood cells with novel *Mtb* infection phase-dependent antigens, including Rv0081, Rv0867c, Rv2389c, Rv1009 and Rv2032 in the diagnosis of TB disease.16,17 However, the 7-day WBA used in that work would not be optimal and useful as a TB diagnostic tool, especially in resource limited settings. Follow-up work evaluated a down selected number of these antigens in the 7-day and overnight cultured assays18 and the present study is a validation of that pilot data.

We enrolled 322 participants with presumed TB and confirmed active disease in 106, whereas active TB was excluded in 216. Comparison of the levels of markers in these two groups, irrespective of their HIV status, and QFT-IT results was performed. Although a sub-group comparison of these markers in the different *Mtb* infection groups was not our primary objective as we were looking for diagnostic tests suitable for the accurate diagnosis of active TB in high endemic settings, with a high prevalence of LTBI, we evaluated the utility of multiple analyte signatures in the diagnosis of TB disease in different HIV and QFT-IT sub groups. We identified several markers that discriminated between latently infected individuals and uninfected groups.

Antigen-specific host markers measured in the overnight WBA in this study did not show much diagnostic potential as the top single markers observed; IFN-γ and IP-10, only achieved an AUC of 64% in discriminating between TB disease and non TB. However, unstimulated levels of SAA, ferritin, CRP and IP-10 were the most promising single markers obtained, reaching AUC ≥70%. As observed in our previous studies16,17 the predictive abilities of these markers improved when they were used in combinations. Indeed, in this study, a six analyte-model showed an improved diagnostic potential. The results of the acute phase proteins: CRP and SAA, are consistent with the results from the pilot study where these markers also featured strongly and were included in the top four-analyte multi marker models.18 In contrast to our previous observations none of VEGF, TGF-α or EGF, which was prominent in the best discriminatory marker model in the 7-day assay, was included into the present models. The larger sample size in the present study and the use of the short term assay are probably responsible for the discrepancy.

Rv0081 is a DosR regulon encoded antigen and several studies have shown that the DosR regulon of *Mtb* is associated with latency, nutrient starvation, hypoxia and low nitric oxide or pH.22-27 Despite the diagnostic potential of this antigen, it did not discriminate between TB and non TB with high sensitivity and specificity and failed to differentiate LTBI from uninfected controls. The evaluation of this antigen in combination with other antigens in previous study did not improve its accuracy.16 Rv0081 elicited tPA responses that were significantly higher in TB cases, in comparison to the non TB group. In contrast to our previous studies, which were conducted in household contacts (HHC) of TB cases, the present study did not recruit contacts as the control group. DosR regulon antigens might be recognised more frequently by people with recent exposure and infection.23, 28-30 IFN-γ elicited by ESAT-6/CFP-10 is a commonly used marker for TB infection and although it does not discriminate between active TB and LTBI on its own, it was included most frequently in the GDA models. This classical antigen also elicited SAA, CRP and ferritin responses. These acute phase proteins are mainly produced in the liver as a result of inflammation and it is not a surprise that these markers, particularly the unstimulated levels, were included in the top analyte models. SAA and CRP are also produced by macrophages and peripheral blood mononuclear cells (PBMCs), respectively,31,32 are being extensively employed as biomarkers in many disease conditions including pulmonary infections.33,34 The potential usefulness of SAA and CRP in serum in the diagnosis of TB has been shown in previous studies46 although no current TB diagnostic tests use these markers.35,36 IP-10 is a chemokine secreted by monocytes with direct interaction with antigen specific T-cells and has been widely researched as an alternative TB immunodiagnostic biomarker.37,38 The levels of stimulated IP-10 was higher in TB disease in our study compared to non TB and this is in agreement with other studies where IP-10 differentiated better between active TB cases and unexposed individuals than IFN-γ release assays (IGRA).39,40 Several studies have shown that the combination of both IFN-γ and IP-10 could significantly enhance diagnostic performance.41-44

The main limitation of our study was the evaluation of fewer antigens than in our previous studies as the down selection of the number of antigens from our pilot work demonstrates the risk for false discovery when a large number of antigens are evaluated in a relatively small number of samples. Antigens can be falsely included or excluded due to insufficient power of the pilot studies. Alternatively, however, the use of shorter term assay here as opposed to the use of long term assays in our previous study might have biased towards responses to a subset of the originally identified antigens only, possibly due to differences in response kinetics. Our results furthermore highlight the fact that multi-marker biosignatures hold promise above the use of single markers. Finally, the results suggest that *ex vivo* samples like plasma and serum may hold promise for the discovery of such biosignatures, as no added accuracy was obtained through stimulation with *Mtb* antigens. We conclude that large future studies should focus on *ex vivo* markers.

**Conclusion**

We identified a biosignature of six unstimulated and mycobacteria-specific host markers in antigen-stimulated overnight WBAs that showed potential in the diagnosis of TB disease with an accurate prediction of 77% TB cases and 84% non TB cases. The sensitivity and specificity of this 6-analyte model was better in HIV uninfected patients but as a large percentage of African TB patients have HIV co-infection, this approach has limited future potential. These markers could, however, be adjunctive markers in the diagnosis of TB disease where sputum is difficult to obtain or where extrapulmonary TB is presumed. Future studies in children and extrapulmonary TB patients should evaluate additional novel *Mtb* antigens, ex-vivo unstimulated markers such as in serum and plasma, and host markers possibly using non-biased approaches such as proteomics to improve sensitivity before field-friendly versions of the stimulation assays are developed.

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