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 studies[16-18] 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 $_{Ag\text{-}Nil,}$ IFN-γ $_{Ag\text{-}Nil,}$ IP-10$_{Nil}$, 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 $_{Ag\text{-}Nil,}$ IFN-γ $_{Ag\text{-}Nil,}$ IP-10$_{Nil}$, 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 assays[18] 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 studies[16,17] 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 studies[46] 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.

Acknowledgements

≠The AE-TBC Consortium: the following present or past members of the consortium contributed to this work.

Stellenbosch University, **South Africa**: Gerhard Walzl, Novel N. Chegou, Magdalena Kriel, Gian van der Spuy, Andre G Loxton, Kim Stanley, Stephanus Malherbe, Shirley Mcanda, Belinda Kriel, Khutso G. Phalane, Paulin Essone

MRC Unit, The Gambia: Jayne S Sutherland, Olumuyiwa Owolabi, Abdou Sillah, Joseph Mendy, Awa Gindeh, Simon Donkor, Toyin Togun, Martin Ota, Awa Gindeh

Karonga Prevention Study, Malawi: Amelia C Crampin, Felanji Simukonda, Alemayehu Amberbir, Femia Chilongo, Rein Houben

Ethiopian Health and Nutrition Research Institute, Ethiopia: Desta Kassa, Atsbeha Gebrezgeabher, Getnet Mesfin, Yohannes Belay, Gebremedhin Gebremichael, Yodit Alemayehu

University of Namibia, Namibia: Marieta van der Vyver, Faustina N Amutenya, Josefina N Nelongo, Lidia Monye, Jacob A Sheehama, Scholastica Iipinge,

Makerere University, Uganda: Harriet Mayanja-Kizza, Ann Ritah Namuganga, Grace Muzanye, Mary Nsereko, Pierre Peters

Armauer Hansen Research Institute, Ethiopia: Rawleigh Howe, Adane Mihret, Yonas Bekele, Bamlak Tessema, Lawrence Yamuah

Leiden University Medical Centre, The Netherlands: Tom H.M. Ottenhoff, Annemieke Geluk, Kees Franken, Paul L.A.M. Corstjens, Elisa M. Tjon Kon Fat, Claudia J. de Dood, Jolien J. van der Ploeg-van Schip

Statens Serum Institut, Copenhagen, Denmark: Ida Rosenkrands, Claus Aagaard

Max Planck Institute for Infection Biology, Berlin, Germany: Stefan H.E. Kaufmann, Maria M. Esterhuyse

London School of Hygiene and Tropical Medicine, London, UK: Jacqueline M. Cliff, Hazel M. Dockrell

Funding

The African European Tuberculosis Consortium (AE-TBC, www.ae-tbc.eu) was funded by the EDCTP, grant number IP_2009_32040), Principal Investigator: Prof. Gerhard Walzl.

Conflict of interest: None

References

1. Flores LL, Steingart KR, Dendukuri N, Schiller I, Minion J, Pai M, et al. Systematic review and meta-analysis of antigen detection tests for the diagnosis of tuberculosis. *Clin Vaccine Immunol* 2011; **18:**1616-1627.
2. Baumann R, Kaempfer S, Chegou NN, Oehlmann W, Loxton AG, Kaufmann SH, et al. Serologic diagnosis of tuberculosis by combining Ig classes against selected mycobacterial targets. *J Infect* 2014; **69:**581-589.
3. Chegou NN, Hoek KG, Kriel M, Warren RM, Victor TC, Walzl G. Tuberculosis assays: past, present and future. *Expert Rev Anti Infect Ther* 2011; **9:**457-469.
4. Verweij KE, Kamerik AR, van Ingen J, van Dijk JH, Sikwangala P, Thuma P, et al. Application of modern microbiological diagnostic methods for tuberculosis in Macha, Zambia. *Int J Tuberc Lung Dis* 2010; **14:**1127-1131.
5. Hussain R, Kaleem A, Shahid F, Dojki M, Jamil B, Mehmood H, et al. Cytokine profiles using whole-blood assays can discriminate between tuberculosis patients and healthy endemic controls in a BCG-vaccinated population. *J Immunol Methods* 2002; **264:**95-108.
6. Flynn JL, Chan J. Immunology of tuberculosis. *Annu Rev Immunol* 2001; **19:**93-129.

348 7. Flynn JL, Chan J. Tuberculosis: latency and reactivation. *Infect Immun* 2001; **69:**4195-
349 4201.
350 8. Collins HL, Kaufmann SH. The many faces of host responses to tuberculosis. *Immunology*
351 2001; **103:**1-9.
352 9. Corstjens PL, Fat, Elisa M Tjon Kon, Claudia J, van der Ploeg-van, Jolien J, Franken KL,
353 Chegou NN, et al. Multi-center evaluation of a user-friendly lateral flow assay to determine
354 IP-10 and CCL4 levels in blood of TB and non-TB cases in Africa. *Clin Biochem* 2016;
355 **49:**22-31.
356 10. Sutherland JS, Mendy J, Gindeh A, Walzl G, Togun T, Owolabi O, et al. Use of lateral
357 flow assays to determine IP-10 and CCL4 levels in pleural effusions and whole blood for
358 TB diagnosis. *Tuberculosis* 2016; **96:**31-36.
359 11. Bobosha K, Fat, Elisa M Tjon Kon, van den Eeden, Susan JF, Bekele Y, van der Ploeg-
360 van, Jolien J, Claudia J, et al. Field-evaluation of a new lateral flow assay for detection of
361 cellular and humoral immunity against Mycobacterium leprae. 2014, 8:e2845.
362 12. Sester M, Sotgiu G, Lange C, Giehl C, Girardi E, Migliori GB, et al. Interferon-gamma
363 release assays for the diagnosis of active tuberculosis: a systematic review and meta-
364 analysis. *Eur Respir J* 2011; **37:**100-111.
365 13. Pai M, Menzies D. The new IGRA and the old TST: making good use of disagreement.
366 *Am J Respir Crit Care Med* 2007; **175:**529-531.
367 14. Kaforou M, Wright VJ, Oni T, French N, Anderson ST, Bangani N, et al. Detection of
368 tuberculosis in HIV-infected and-uninfected African adults using whole blood RNA
369 expression signatures: a case-control study. 2013; **10:**e1001538.
370 15. Ottenhoff TH. New pathways of protective and pathological host defense to
371 mycobacteria. *Trends Microbiol* 2012; **20:**419-428.
372 16. Chegou NN, Black GF, Loxton AG, Stanley K, Essone PN, Klein MR, et al. Potential of
373 novel Mycobacterium tuberculosis infection phase-dependent antigens in the diagnosis
374 of TB disease in a high burden setting. *BMC Infect Dis* 2012; **12:**10.
375 17. Chegou NN, Essone PN, Loxton AG, Stanley K, Black GF, Van der Spuy, Gian D, et al.

Potential of host markers produced by infection phase-dependent antigen-stimulated cells for the diagnosis of tuberculosis in a highly endemic area. *PLoS One* 2012; **7:**e38501.

18. Essone PN, Chegou NN, Loxton AG, Stanley K, Kriel M, van der Spuy G, et al. Host cytokine responses induced after overnight stimulation with novel M. tuberculosis infection phase-dependent antigens show promise as diagnostic candidates for TB disease. *PLoS One* 2014; **9:**e102584.
19. Chegou NN, Black GF, Kidd M, van Helden PD, Walzl G. Host markers in QuantiFERON supernatants differentiate active TB from latent TB infection: preliminary report. *BMC Pulm Med* 2009; **9:**21.
20. Betts JC, Lukey PT, Robb LC, McAdam RA, Duncan K. Evaluation of a nutrient starvation model of Mycobacterium tuberculosis persistence by gene and protein expression profiling. *Mol Microbiol* 2002; **43:**717-731.
21. Commandeur S, van den Eeden, Susan JF, Dijkman K, Clark SO, van Meijgaarden KE, Wilson L, et al. The in vivo expressed Mycobacterium tuberculosis (IVE-TB) antigen Rv2034 induces CD4 T-cells that protect against pulmonary infection in HLA-DR transgenic mice and guinea pigs. *Vaccine* 2014; **32:**3580-3588.
22. Zvi A, Ariel N, Fulkerson J, Sadoff JC, Shafferman A. Whole genome identification of Mycobacterium tuberculosis vaccine candidates by comprehensive data mining and bioinformatic analyses. *BMC Med Genomics* 2008; **1:**18.
23. Leyten EM, Lin MY, Franken KL, Friggen AH, Prins C, van Meijgaarden KE, et al. Human T-cell responses to 25 novel antigens encoded by genes of the dormancy regulon of Mycobacterium tuberculosis. *Microb Infect* 2006; **8:**2052-2060.
24. Honaker RW, Stewart A, Schittone S, Izzo A, Klein MR, Voskuil MI. Mycobacterium bovis BCG vaccine strains lack narK2 and narX induction and exhibit altered phenotypes during dormancy. *Infect Immun* 2008; **76:**2587-2593.
25. Wayne LG, Sohaskey CD. Nonreplicating Persistence of Mycobacterium Tuberculosis 1 . *Annu Rev Microbiol* 2001; **55:**139-163.

404 26. Lin MY, Ottenhoff TH. Not to wake a sleeping giant: new insights into host-pathogen
405 interactions identify new targets for vaccination against latent Mycobacterium
406 tuberculosis infection. *Biol Chem* 2008; **389:**497-511.
407 27. Roupie V, Romano M, Zhang L, Korf H, Lin MY, Franken KL, et al. Immunogenicity of
408 eight dormancy regulon-encoded proteins of Mycobacterium tuberculosis in DNA-
409 vaccinated and tuberculosis-infected mice. *Infect Immun* 2007; **75:**941-949.
410 28. Black GF, Thiel BA, Ota MO, Parida SK, Adegbola R, Boom WH, et al. Immunogenicity
411 of novel DosR regulon-encoded candidate antigens of Mycobacterium tuberculosis in
412 three high-burden populations in Africa. *Clin Vaccine Immunol* 2009; **16:**1203-1212.
413 29. Commandeur S, Lin MY, van Meijgaarden KE, Friggen AH, Franken KL, Drijfhout JW, et
414 al. Double-and monofunctional CD4 and CD8 T-cell responses to Mycobacterium
415 tuberculosis DosR antigens and peptides in long-term latently infected individuals. *Eur J*
416 *Immunol* 2011; **41:**2925-2936.
417 30. Schuck SD, Mueller H, Kunitz F, Neher A, Hoffmann H, Franken K, et al. Identification of
418 T-cell antigens specific for latent Mycobacterium tuberculosis infection. *PloS one* 2009;
419 **4:**e5590.
420 31. Meek RL, Urieli-Shoval S, Benditt EP. Expression of apolipoprotein serum amyloid A
421 mRNA in human atherosclerotic lesions and cultured vascular cells: implications for
422 serum amyloid A function. *Proc Natl Acad Sci* U S A 1994; **91:**3186-3190.
423 32. Haider D, Leuchten N, Schaller G, Gouya G, Kolodjaschna J, Schmetterer L, et al.
424 C-reactive protein is expressed and secreted by peripheral blood mononuclear cells.
425 *ClinExp Immunol* 2006; **146:**533-539.
426 33. Fujiwara H, Suchi K, Okamura S, Okamura H, Umehara S, Todo M, et al. Elevated
427 serum CRP levels after induction chemoradiotherapy reflect poor treatment response in
428 association with IL-6 in serum and local tumor site in patients with advanced esophageal
429 cancer. *J Surg Oncol* 2011; **103:**62-68.
430 34. dos Anjos BL, Grotto HZ. Evaluation of C-reactive protein and serum amyloid A in the
431 detection of inflammatory and infectious diseases in children. *Clin Chem Lab Med* 2010;

48:493-499.

35. Phalane KG, Kriel M, Loxton AG, Menezes A, Stanley K, Van der Spuy, Gian D, et al. Differential expression of host biomarkers in saliva and serum samples from individuals with suspected pulmonary tuberculosis. *Mediators Inflamm* 2013; 2013.
36. Maasilta P, Kostiala AA. Serum levels of C-reactive protein in patients with pulmonary tuberculosis and malignant tumors of the chest. *Infection* 1989; **17:**13-14.
37. Sauty A, Dziejman M, Taha RA, Iarossi AS, Neote K, Garcia-Zepeda EA, et al. The T cell-specific CXC chemokines IP-10, Mig, and I-TAC are expressed by activated human bronchial epithelial cells. *J Immunol* 1999; **162:**3549-3558.
38. Chegou NN, Heyckendorf J, Walzl G, Lange C, Ruhwald M. Beyond the IFN-gamma horizon: biomarkers for immunodiagnosis of infection with Mycobacterium tuberculosis. *Eur Respir J* 2014; **43:**1472-1486.
39. Hong JY, Jung GS, Kim H, Kim YM, Lee HJ, Cho S, et al. Efficacy of inducible protein 10 as a biomarker for the diagnosis of tuberculosis. *Int J Infect Dis* 2012; **16:**e855-e859.
40. Syed Ahamed Kabeer B, Paramasivam P, Raja A. Interferon gamma and interferon gamma inducible protein-10 in detecting tuberculosis infection. *J Infect* 2012; **64:**573-579.
41. Aabye MG, Ruhwald M, Praygod G, Jeremiah K, Faurholt-Jepsen M, Faurholt-Jepsen D, et al. Potential of interferon-gamma-inducible protein 10 in improving tuberculosis diagnosis in HIV-infected patients. *Eur Respir J* 2010; **36:**1488-1490.
42. Kabeer BSA, Raman B, Thomas A, Perumal V, Raja A. Role of QuantiFERON-TB gold, interferon gamma inducible protein-10 and tuberculin skin test in active tuberculosis diagnosis. *PLoS One* 2010; **5:**e9051.
43. Ruhwald M, Dominguez J, Latorre I, Losi M, Richeldi L, Pasticci MB, et al. A multicentre evaluation of the accuracy and performance of IP-10 for the diagnosis of infection with M. tuberculosis. *Tuberculosis* 2011; **91:**260-267.
44. Ruhwald M, Bodmer T, Maier C, Jepsen M, Haaland MB, Eugen-Olsen J, et al. Evaluating the potential of IP-10 and MCP-2 as biomarkers for the diagnosis of

tuberculosis. *Eur Respir J* 2008; **32:**1607-1615.

45. Jacobs R, Tshehla E, Malherbe S, Kriel M, Loxton AG, Stanley K, et al. Host markers detected in saliva show promise as markers for the diagnosis of pulmonary tuberculosis disease and monitoring of the response to tuberculosis treatment *Cytokine* 2016*;* **81:**50-56.

46. Chegou NN, Sutherland JS, Malherbe S, Crampin AC, Corstjens PLAM, Geluk A, et al. Diagnostic Performance of a Seven-marker Serum Protein Biosignature for the Diagnosis of Active TB Disease in African Primary Health Care Clinic Attendees with Signs and Symptoms Suggestive of TB. ***Thorax*** 2016 (Article in press)