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1 **Introduction**

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

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

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

28 of some of these promising antigens to elicit a host response in a short term (overnight)
29 whole blood assay compared to the long term (7 day) whole blood assay.¹⁸ This study also
30 evaluated the accuracy of some of these previously reported novel candidate antigens but in
31 a larger study employing a short term (overnight), more field-friendly whole blood assay.

32 **Materials and methods**

33 **Study participants**

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

55 described.¹⁹ The Human Ethics Research Committee of the University of Stellenbosch gave
56 approval for the study (N10/08/274).

57 **Reference standard for classification of study participants**

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

70

71 **Whole blood culture assay (WBA)**

72 At enrolment, 10ml of heparinised blood was collected from all participants and transported
73 at ambient conditions within two hours of collection to the laboratory where the WBA was
74 performed. The antigens that were used came from two sources namely: Leiden University
75 Medical Center (LUMC), The Netherlands, and the Statens Serum Institut (SSI), Denmark.
76 ESAT-6 and CFP-10 are two separate antigens, but were measured together as a fusion
77 protein (ESAT-6/CFP-10) in this study. ESAT-6/CFP-10 and RV0081 were selected for the
78 current study because of the promising accuracy shown by host markers elicited by these
79 antigens in our previous studies¹⁶⁻¹⁸ whereas Rv1284 and Rv2034 were selected because of
80 the promise already shown by the antigens as TB diagnostic and vaccine candidates in
81 previous studies.^{20,21} Prior to their usage the four lyophilised antigens were reconstituted in

82 sterile 1X PBS. The reconstituted antigens were then diluted in sterile 1x PBS, mixed with
83 undiluted whole blood from each study participant at a final concentration of 10µg/ml, and
84 incubated overnight (20-24hours) in 24-well tissue culture plates (Corning Corstar, Sigma)
85 as previously described.¹⁸ Sterile 1x PBS (Lonza, Cat #: 17-517Q) was used as the negative
86 control.

87 **Luminex multiplex immunoassay**

88 This prospective study included 322 TB and non TB cases and was evaluated using a
89 Luminex multiplex cytokine platform which is based on simultaneous detection and
90 cytometric quantification of different cytokines in a sample. The concentrations of 42 host
91 markers including interleukin (IL)-1β, IL-1Rα, IL-2, IL-4, IL-5, IL-6, IL-7, IL-8, IL-9, IL-10, IL-
92 12, IL-13, IL-15, IL-17, eotaxin, fibroblast growth factor (FGF) basic, granulocyte colony
93 stimulating factor (G-CSF), granulocyte monocyte colony stimulating factor (GM-CSF), IFN-
94 γ, interferon inducible protein (IP)-10, monocyte chemotactic protein (MCP)-1, macrophage
95 inflammatory protein (MIP)-1α, platelet derived growth factor BB (PDGF-BB), MIP-1β,
96 RANTES, TNF-α, vascular endothelial growth factor (VEGF), eotaxin-2, BCA-1, 6Ckine,
97 SCF, TRAIL, ENA, ferritin, fibrinogen, procalcitonin, serum amyloid protein A (SAA), tissue
98 plasminogen activator, serum amyloid protein P (SAP), CRP, haptoglobin and α-2
99 macroglobulin, were evaluated in WBA supernatants of all the study participants. This was
100 done using Milliplex kits (Merck Millipore, St. Charles, Missouri, USA) and Bio-Plex kits (Bio
101 Rad Laboratories, Hercules, CA, USA) on the Bio-Plex™ platform according to the
102 manufacturer's instructions. Standard curves were generated from the serial dilutions that
103 were made from the assay controls supplied and matched against the cytokine concentration
104 for quantification. The concentrations of all the analytes in the quality control reagents were
105 found to be within the ranges as expected. The Bio-Plex manager version 6.1 was used for
106 bead acquisition and analysis of median fluorescence intensity.

107

108

109 **Statistical analysis**

110 Statistical differences in analyte levels were evaluated by the Mann Whitney U test for non-
111 parametric data analysis. The diagnostic accuracies of individual antigen-specific or
112 unstimulated responses for TB disease were ascertained by receiver operator characteristics
113 (ROC) curve analysis. Cut-off levels for estimation of sensitivity and specificity were selected
114 based on the Youden's Index. The predictive abilities of combinations of unstimulated and
115 antigen-specific host markers for TB disease and non TB were investigated by performing
116 best subsets general discriminant analysis (GDA). Data were randomly partitioned into a
117 70% training data set, which was used for model building and 30% test set, which was used
118 to verify the accuracy of the different models. The leave-one-out cross validation approach
119 was used to test the prediction accuracy of biosignatures after data was stratified according
120 to HIV status, due to the relatively limited number of HIV infected individuals. Data were
121 analyzed using GraphPad prism, version 5.00 for Windows (Graphpad Software, San Diego
122 California, USA) and Statistica (Statsoft, Ohio, USA).

123 **Results**

124 **Study participants**

125
126 A total of 322 participants were enrolled into this study, 106 (33%) of who were cultures
127 positive TB cases (Figure 1). Of the 322 study participants, 168 (52%) were males and 24
128 (23%) of the 106 TB cases were HIV co-infected. The demographic and clinical information
129 of the participants are shown in table 1.

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

132 When the analyte levels detected in the unstimulated control supernatants in TB patients
133 were compared to the levels obtained in the non TB group (50% of this group were QFT-IT
134 positive), the unstimulated levels of 14 out of the 42 host markers evaluated showed
135 significant differences. The concentrations of these markers including CRP, Ferritin, IP-10,
136 IL-6, IL-7, IL-9, IL-13, IFN- γ , VEGF, Haptoglobin, SAP, PCT and SAA were significantly

137 higher in the TB group (Table 2). When the diagnostic potentials of these unstimulated host
138 markers were evaluated by ROC curve analysis, four analytes including CRP, IP-10, Ferritin
139 and SAA had an area under the ROC curve (AUC) of ≥ 0.85 , ≥ 0.74 , ≥ 0.79 and ≥ 0.77
140 respectively, in unstimulated samples. At their optimal unstimulated cut-off values, SAA had
141 a sensitivity and specificity of 81% and 72%, ferritin 70% for both sensitivity and specificity,
142 IP-10 had 77% sensitivity and 71% specificity for ascertaining TB disease. The best
143 performance characteristic was with unstimulated CRP with a sensitivity and specificity of
144 80% (Table 2, Figure 3). The high AUC recorded for some of these markers support their
145 diagnostic potential.

146 **Utility of host markers detected in overnight antigen-stimulated culture** 147 **supernatants in the diagnosis of TB disease**

148 The unstimulated control levels for the different host markers were subtracted from the
149 antigen-stimulated responses for each study participant before the analysis of the data. In
150 response to *Mtb*-specific antigenic stimulation by ESAT-6/CFP-10, median concentrations of
151 IP-10, IFN- γ , IL-1R α , tPA and TRAIL were significantly higher in the TB group ($p < 0.05$)
152 (Table 2, Figure 2). Following stimulation with Rv2034, IL-2, IL-17 and FGF basic levels
153 were significantly higher in TB cases whereas ferritin was higher in non TB. Rv1284 elicited
154 the production of significantly high levels of IL-2 in the non TB cases, whereas only tPA
155 responses were significantly different between the TB and non TB cases after stimulation
156 with Rv0081 (Table 2). When the diagnostic accuracy of individual antigen-specific host
157 markers were investigated by ROC curve analysis, the AUCs for ESAT-6/CFP-10 stimulated
158 IP-10 and IFN- γ were ≥ 0.64 respectively. Antigen-specific level of IP-10 had the best
159 sensitivity of 60% and specificity of 65%. The AUC's of Rv1284-specific and Rv2034-specific
160 markers performed poorly in general. Only Rv2034-specific level of IL-2 attained 0.60 (Table
161 2, Figure 3).

162

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

165 When the concentration of host markers detected in QFT-IT positive non TB cases (LTBI)
166 were compared to the levels obtained in the QFT-IT negative non TB cases (uninfected
167 controls), the unstimulated levels of IL-1 β , IL-1R α , IL-6, IL-10, IL-12, MIP-1 α , TNF- α and
168 were significantly higher in the uninfected controls. Only unstimulated levels of eotaxin were
169 significantly higher in LTBI subjects. When the host markers elicited after stimulation with the
170 different antigens were compared between the two groups, most of the discriminatory
171 markers were found in ESAT-6/CFP-10 stimulated supernatants. ESAT-6/CFP-10 -specific
172 levels of IL-1R α , IL-2, IL-4, IL-5, IL-13, IL-15, FGF basic, GM-CSF, IFN- γ , IP-10, MCP-1,
173 MIP-1 α and Eotaxin-2 were significantly higher in the LTBI group. Similarly, Rv2034-specific
174 levels of IL-8, IL-15, MCP-1 and MIP-1 α , and Rv1284-specific levels of G-CSF, MCP-1 and
175 PDGF-BB were significantly higher in the LTBI. Stimulation with Rv0081 failed to elicit any
176 response. When the diagnostic accuracies of the markers detected in the culture
177 supernatants were evaluated by ROC curve analysis, only ESAT-6/CFP-10-specific levels of
178 IP-10, IFN- γ , GM-CSF, IL-2 and IL-13 discriminated between the two groups with AUC \geq
179 0.70. Out of these five markers, ESAT-6/CFP-10-specific level of IP-10 had the best
180 sensitivity and specificity of 75% and 72% respectively. Although ESAT-6/CFP-10-specific
181 IL-5 and eotaxin-2, Rv2034-specific MCP-1, and Rv1284-specific PDGF-BB all discriminated
182 between the two groups with sensitivities $>80\%$, the specificities of all these markers were
183 poor, ranging between 38-50% (Table 3).

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

186 To evaluate the predictive abilities of combinations of analytes for TB and no TB disease
187 data obtained from all study participants were analysed by general discriminant analysis
188 (GDA), regardless of the HIV infection status of the study participants. The unstimulated and
189 antigen-specific responses of each host marker were treated as separate variables, in order

190 to evaluate the contribution of both classes of markers in predictive models. We randomly
191 partitioned all the data from the measurement of the different markers into a 70% training
192 data set for model building, and 30% for a test set for the verification of the models. A
193 combination of six markers IP-10_{Ag-Nil}, IFN- γ _{Ag-Nil}, IP-10_{Nil}, Ferritin_{Nil}, SAA_{Nil}, and CRP_{Nil}
194 accurately predicted 77% TB cases and 84% of the non TB cases in the training set,
195 regardless of HIV infection status. In the test set, the six-marker biosignature accurately
196 predicted 83% of the TB cases and 78% of the non TB cases (Table 4).

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

208 **Discussion**

209 The development of a new, relatively rapid, and accurate test, that does not rely on sputum,
210 which can be difficult to obtain in some patient groups, and which does not reflect the site of
211 infection in extrapulmonary TB, would be a major advance in the TB diagnostic field. The
212 measurement of a small number of analytes that differentiates active TB from LTBI in the
213 blood in a short-term overnight assay, might fulfil this need.¹⁹ Test results would be available
214 within 48 hours, rather than after several weeks as is the case with sputum culture. In this
215 study we investigated the potential accuracy of host markers detected in supernatants, after
216 stimulation of whole blood with *Mtb* infection phase-dependent antigens, in an overnight

217 culture assay. We have shown that multiple biomarkers detected in the antigen-stimulated
218 and unstimulated supernatants can contribute to a diagnostic signature with the ability to
219 discriminate between active TB and non TB. A biosignature of six analytes showed
220 promising results especially in HIV uninfected individuals. We previously reported on the
221 potential of host markers produced after stimulation of blood cells with novel *Mtb* infection
222 phase-dependent antigens, including Rv0081, Rv0867c, Rv2389c, Rv1009 and Rv2032 in
223 the diagnosis of TB disease.^{16,17} However, the 7-day WBA used in that work would not be
224 optimal and useful as a TB diagnostic tool, especially in resource limited settings. Follow-up
225 work evaluated a down selected number of these antigens in the 7-day and overnight
226 cultured assays¹⁸ and the present study is a validation of that pilot data.

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

236 Antigen-specific host markers measured in the overnight WBA in this study did not show
237 much diagnostic potential as the top single markers observed; IFN- γ and IP-10, only
238 achieved an AUC of 64% in discriminating between TB disease and non TB. However,
239 unstimulated levels of SAA, ferritin, CRP and IP-10 were the most promising single markers
240 obtained, reaching AUC $\geq 70\%$. As observed in our previous studies^{16,17} the predictive
241 abilities of these markers improved when they were used in combinations. Indeed, in this
242 study, a six analyte-model showed an improved diagnostic potential. The results of the acute
243 phase proteins: CRP and SAA, are consistent with the results from the pilot study where

244 these markers also featured strongly and were included in the top four-analyte multi marker
245 models.¹⁸ In contrast to our previous observations none of VEGF, TGF- α or EGF, which was
246 prominent in the best discriminatory marker model in the 7-day assay, was included into the
247 present models. The larger sample size in the present study and the use of the short term
248 assay are probably responsible for the discrepancy.

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

271 levels of stimulated IP-10 was higher in TB disease in our study compared to non TB and
272 this is in agreement with other studies where IP-10 differentiated better between active TB
273 cases and unexposed individuals than IFN- γ release assays (IGRA).^{39,40} Several studies
274 have shown that the combination of both IFN- γ and IP-10 could significantly enhance
275 diagnostic performance.⁴¹⁻⁴⁴

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

288 **Conclusion**

289 We identified a biosignature of six unstimulated and mycobacteria-specific host markers in
290 antigen-stimulated overnight WBAs that showed potential in the diagnosis of TB disease with
291 an accurate prediction of 77% TB cases and 84% non TB cases. The sensitivity and
292 specificity of this 6-analyte model was better in HIV uninfected patients but as a large
293 percentage of African TB patients have HIV co-infection, this approach has limited future
294 potential. These markers could, however, be adjunctive markers in the diagnosis of TB
295 disease where sputum is difficult to obtain or where extrapulmonary TB is presumed. Future
296 studies in children and extrapulmonary TB patients should evaluate additional novel *Mtb*
297 antigens, ex-vivo unstimulated markers such as in serum and plasma, and host markers

298 possibly using non-biased approaches such as proteomics to improve sensitivity before field-
299 friendly versions of the stimulation assays are developed.

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332

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