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1 **Title page**

2 **Title**

3 Blood RNA biomarkers for tuberculosis screening in people living with HIV prior to anti-retroviral therapy
4 initiation: A diagnostic accuracy study.

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Abstract

Background

Undiagnosed tuberculosis (TB) remains a major threat for people living with HIV (PLHIV). Multiple blood transcriptomic biomarkers have shown promise for TB diagnosis. We sought to evaluate their diagnostic accuracy and clinical utility for systematic pre-antiretroviral therapy (ART) TB screening.

Methods

We enrolled consecutive adults referred to start ART at a community health centre in Cape Town, South Africa, irrespective of symptoms. Sputa were obtained (using induction if required) for two liquid cultures. Whole-blood RNA samples underwent transcriptional profiling using a custom Nanostring gene-panel. We measured the diagnostic accuracy of seven candidate RNA biomarkers for the reference standard of *Mycobacterium tuberculosis* culture status, using area under the receiver-operating characteristic curve (AUROC) analysis, and sensitivity/specificity at pre-specified thresholds (two standard scores above the mean of healthy controls; Z2). Clinical utility was assessed using decision curve analysis. We compared performance to CRP (threshold ≥5mg/L), World Health Organisation (WHO) four-symptom screen (W4SS) and the WHO target product profile for TB triage tests.

Results

A total of 707 PLHIV were included, with median CD4 count 306 cells/mm3. Of 676 with available sputum culture results, 89 (13%) had culture-confirmed TB. The seven RNA biomarkers were moderately to highly correlated (Spearman rank coefficients 0.42-0.93) and discriminated TB culture-positivity with similar AUROCs (0.73-0.80), but none statistically better than CRP (AUROC 0.78; 95% CI 0.72-0.83). Diagnostic accuracy was similar across CD4 count strata, but lower among W4SS-negative (AUROCs 0.56-0.65) compared to W4SS-positive participants (AUROCs 0.75-0.84). The RNA biomarker with highest AUROC point estimate was a 4-gene signature (Suliman4; AUROC 0.80; 95% CI 0.75-0.86), with sensitivity 0.83 (0.74-0.90) and specificity 0.59 (0.55-0.63) at Z2 threshold. In decision curve analysis, Suliman4 and CRP had similar clinical utility to guide confirmatory TB testing, but both had higher net benefit than W4SS. In exploratory analyses, an approach combining CRP (≥5mg/L) and Suliman4 (≥Z2) had sensitivity of 0.80 (0.70-0.87), specificity of 0.70 (0.66-0.74) and higher net benefit than either biomarker alone.

Interpretation

RNA biomarkers showed better clinical utility to guide confirmatory TB testing for PLHIV prior to ART initiation than symptom-based screening, but their performance did not exceed that of CRP, and fell short of WHO recommended targets. Interferon-independent approaches may be required to improve accuracy of host-response biomarkers to support TB screening pre-ART initiation.

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61 **Research in Context**

62 **Evidence before this study**

63 The World Health Organisation (WHO) commissioned a recent systematic review and individual participant
64 data meta-analysis of tuberculosis (TB) screening strategies among ambulatory people living with HIV (PLHIV).
65 TB is a major cause of morbidity and mortality among PLHIV, particularly among those with untreated HIV and
66 consequent immunosuppression. Importantly, initiation of antiretroviral treatment (ART) for HIV is also
67 associated with increased short-term risk of incident TB, attributed to immune reconstitution inflammatory
68 syndrome, which may in turn potentiate the immunopathogenesis of TB. As a result, in high TB prevalence
69 settings, systematic screening for TB is widely advocated for PLHIV before starting ART. In this context,
70 universal sputum microbiological screening is not economically sustainable, and limited by practical feasibility
71 among those who are not expectorating sputum. Patient stratification to identify those at greater risk of TB is
72 required to target resources for microbiological testing more precisely. For this purpose, the WHO four
73 symptom screen (W4SS) achieved an estimated 84% sensitivity and 37% specificity for pre-ART TB screening.
74 Blood CRP ≥5mg/L offered better performance, estimated at 89% sensitivity and 54% specificity respectively,
75 but still fell short of the WHO target product profile, aiming for ≥90% sensitivity and ≥70% specificity. Blood
76 RNA biomarkers of TB, reflecting interferon (IFN) and tumour necrosis factor-mediated immune responses,
77 have been gaining momentum as potential triage tests for symptomatic and pre-symptomatic TB, but their
78 performance has not been comprehensively evaluated among PLHIV initiating ART. Untreated HIV also drives
79 chronic IFN activity that may compromise the specificity of IFN-dependent biomarkers in this population.

80 **Added value of this study**

81 To our knowledge, this is the largest study to date to benchmark the performance of candidate blood RNA
82 biomarkers for unselected and systematic pre-ART TB screening among PLHIV, against contemporary
83 standards and aspirational performance targets. The blood RNA biomarkers showed better diagnostic
84 accuracy and clinical utility to guide confirmatory TB testing for PLHIV than symptom-based screening with
85 W4SS, but their performance did not exceed that of CRP, and they did not achieve WHO recommended
86 targets. The results were comparable for microbiologically confirmed TB at enrolment to the study and for all
87 cases starting TB treatment within six months of enrolment. Blood RNA biomarkers correlated with features of
88 disease severity that might be attributed to either TB or HIV. Accordingly, their discrimination of TB among
89 PLHIV was particularly limited by poor specificity. Diagnostic accuracy was significantly better among people
90 who were symptomatic compared to those who were asymptomatic, further limiting the value of RNA
91 biomarkers in pre-symptomatic TB. Interestingly, blood RNA biomarkers only showed moderate correlation
92 with CRP, suggesting these two measurements provided information on different components of the host
93 response. An exploratory analysis showed that CRP can be combined with the best performing blood RNA
94 signature to provide better clinical utility than achieved by either test alone.

95 **Implications of all the available evidence**

96 Our data demonstrate that blood RNA biomarkers do not perform any better than CRP as triage tests for TB
97 among PLHIV prior to ART initiation. Since CRP is already widely available on a low cost point-of-care platform,
98 our findings support further evaluation of the clinical and health-economic impact of CRP-based triage for pre-
99 ART TB screening. An underlying mechanism that limits the diagnostic accuracy of RNA biomarkers for TB

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100 among PLHIV prior to ART may be upregulation of interferon signalling in untreated HIV. Since interferon
101 activity underpins upregulated expression of TB biomarker genes, HIV-induced upregulation of interferon-
102 stimulated genes may reduce the specificity of blood transcriptomic biomarkers for TB in this context. These
103 findings highlight a wider need to identify interferon-independent host-response based biomarkers to support
104 disease specific screening of PLHIV pre-ART initiation.

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105 **Introduction**

106 Tuberculosis (TB) continues to be a leading cause of death among people living with HIV (PLHIV) and often
107 remains undiagnosed[1,2]. Prompt diagnosis and treatment initiation are required to reduce mortality in HIV-
108 associated TB (HIV-TB). However, diagnosis can be challenging, with only 56% of estimated incident HIV-TB
109 cases notified in 2019[3]. The World Health Organization (WHO) therefore recommends systematic screening
110 for TB among PLHIV at every healthcare encounter[3]. Since 2011, systematic screening has been based on
111 the WHO four-symptom screen (W4SS) to trigger confirmatory TB testing using culture or molecular assays,
112 and investigate for TB prior to initiation of preventative treatment[3]. W4SS achieves an estimated 84%
113 sensitivity and 37% specificity for TB among outpatients not receiving anti-retroviral therapy (ART)[4]. More
114 recently, WHO have also endorsed measurement of blood C-reactive protein ≥5 mg/L (CRP) for this
115 application, offering similar sensitivity (89%) but higher specificity (54%) than W4SS in an individual participant
116 data meta-analysis[3,4]. However, this still falls short of their target product profile (TPP) which aims to achieve
117 ≥90% sensitivity and ≥70% specificity[5], necessitating the development of novel approaches with greater
118 accuracy than W4SS or CRP[34].

119 Multiple blood transcriptomic biomarkers for TB have shown promise for prediction of incident TB over a 3-6
120 month interval[6] and for diagnosis of prevalent TB among symptomatic people[7–9]. Head-to-head analyses have
121 demonstrated areas under the receiver operating characteristic curves (AUROCs) of 0.87-0.91 for the best
122 performing signatures to identify culture-positive TB among symptomatic people self-presenting to healthcare
123 services, irrespective of HIV status[7]. In the context of general population screening, transcriptomic signature
124 AUROCs for prevalent TB have been estimated as 0.63-0.79 in HIV-uninfected people and 0.65-0.88 among
125 PLHIV, with inferior performance among asymptomatic people[8,10]. Signatures are now being translated to near-
126 patient assays, for example using the GeneXpert platform, with promising initial performance among
127 symptomatic individuals[11]. However, data among PLHIV are scarce with only 10 prevalent TB cases in the
128 only previous general population screening study of PLHIV[12]. Moreover, the vast majority of PLHIV in previous
129 studies of both passive and active-case finding were receiving ART. Data from untreated PLHIV are distinctly
130 lacking.

131 Pre-antiretroviral therapy (ART) initiation is a key timepoint for TB screening, since this frequently represents
132 a nadir of immunocompromise associated with elevated risk of TB both before and during the initial months of
133 ART[13]. Untreated HIV may also be associated with upregulation of interferon signalling[12,14,15] that may reduce
134 the specificity of blood transcriptomic biomarkers for TB, as also demonstrated during intercurrent respiratory
135 viral infections[16,17]. In this study, we sought to evaluate the diagnostic accuracy and clinical utility of a range of
136 concise candidate blood transcriptomic signatures for TB in a large cohort of PLHIV, in South Africa, prior to
137 commencement of ART. We benchmarked performance against the WHO TPP, along with CRP and W4SS
138 as alternative screening approaches.

139 **Methods**

140 **Study cohort**

141 Consecutive adults with HIV infection referred to start ART at Kraaifontein Community Health Centre in Cape
142 Town, South Africa, were prospectively enrolled in a parent study evaluating the diagnostic accuracy of sputum
143 Xpert Mtb/Rif Ultra (hereafter, Ultra), irrespective of symptoms. Exclusion criteria included TB treatment within
144 two months or unknown current TB treatment status. In the current study, we included participants who were

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145 recruited between 15 May 2017 and 14 December 2020 and who had blood RNA and CRP data available
146 (Figure 1). This study was approved by the Stellenbosch University Faculty of Health Sciences Research
147 Ethics Committee (N14/10/136) and the Western Cape Department of Health, South Africa
148 (WC_2016RP38_944), and is registered on clinicaltrials.gov (NCT03187964). The study is reported in line with
149 STARD guidance[18].

150 Demographic, co-morbidity, symptom and TB treatment data were captured at baseline. CRP was measured
151 in real-time using a point-of-care assay (iChromaII platform, Boditech, South Korea), or a retrospective
152 laboratory assay using stored serum when point-of-care data were unavailable. Three sputum samples were
153 obtained per participant; two underwent smear microscopy and liquid culture and the third sample was tested
154 using Ultra. Expectoration was attempted for at least one sample. If this was not sufficient, sputum induction
155 was performed using nebulised hypertonic saline. Blood RNA was collected in Tempus tubes (Ambion, Life
156 Technologies) and preserved at -80ºC. Urine samples were collected and tested using Ultra and Determine
157 LF-LAM (Abbott, South Africa). TB diagnoses and treatment data after enrolment were obtained through
158 linkage to routinely collected health record data held at the Western Cape Provincial Health Data Centre using
159 a deterministic algorithm[19].

160 The reference standard for primary analyses was sputum liquid culture status for *Mycobacterium tuberculosis*
161 *complex*. Secondary reference standards included: (1) sputum culture *or* Ultra positivity (excluding trace
162 positive results); (2) any positive TB test, including urine LAM or Ultra (excluding sputum Ultra trace positive
163 results); and (3) recorded TB diagnosis or TB treatment in study or linkage data within six months of enrolment.
164 Participants with missing blood RNA or outcome data were excluded for each analysis.

165 Our sample size calculation was based on achieving a minimum AUROC of 0.8, equivalent to that of CRP[4]
166 with a 95% lower confidence interval bound of 0.75, requiring 700 participants with a minimum TB prevalence
167 rate of 10% (Supplementary Figure 1)[20].

168 **Sample processing and Nanostring**

169 Peripheral blood RNA samples were extracted using the Tempus Spin RNA Isolation kit (Ambion, Life
170 Technologies) and the Turbo DNA-free Kit (Invitrogen). RNA integrity scores were determined using the Agilent
171 Tape Station (Agilent). Transcriptional profiling of 300ng of blood RNA was performed using a custom gene
172 panel on the Nanostring platform (Nanostring Technologies). The gene panel was designed to include 23 TB
173 genes encompassing seven candidate RNA signatures for TB, limited to concise signatures of ≤11 genes that
174 performed well in our previous head-to-head analyses[7,21]. The signatures included were Suliman4, RISK6,
175 Sweeney3, Giddon3, BATF2, Roe3 and Zak11. Signatures are referred to with a prefix of the first-author's
176 surname from the original publication where the signature was derived, and a suffix of the number of
177 component genes, with the exceptions of BATF2 (a single transcript) and RISK6 (as named by the original
178 investigators)[22].

179 Nanostring data were analysed on the nCounter Analyser (Nanostring Technologies) using 555 field of view,
180 as per manufacturer's instructions. Quality control and gene expression normalisation were performed with
181 nSolver Analysis software (version 4.0.70). Gene expression values were log2-transformed and then
182 normalised by subtracting the log2-expression of a housekeeping gene (*GAPDH*). Blood RNA signature Z-
183 scores were calculated by standardising scores for each signature to the mean and standard deviation of blood

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184 samples (N=105) from a healthy control population of individuals with latent TB[23], also measured using the
185 same Nanostring codeset.

186 Reference RNA samples (Universal Human Reference RNA, Agilent) were included in each Nanostring run to
187 facilitate quality control. These demonstrated minimal coefficients of variation for each gene, supporting
188 reproducibility of measurements (Supplementary Figure 2). We also examined the discrimination of TB and
189 non-TB cases using Nanostring measurements of the seven candidate RNA signatures in 59 paired RNA
190 samples from our previously reported presumptive TB cohort[7], to ensure that we could reproduce the results
191 derived from RNAseq data (Supplementary Figure 3).

192 Principal component analyses revealed systematic differences in reference RNA data by manufacturing
193 codeset batch (Supplementary Figure 4). We therefore performed batch correction by codeset manufacturing
194 batch, using the *ComBat* function from the *sva* package in R[24]. Distributions of target genes differed by batch
195 to a varying degree for each probe prior to batch correction (Supplementary Figure 5). These differences
196 resolved after correction (Supplementary Figure 6-7).

197 **Data analysis**

198 Analyses were conducted R (version 4.0.2). Blood RNA signature scores were calculated from processed
199 Nanostring data as reported previously (Supplementary Table 1)[7,21]. For the Roe3 signature, we sought to
200 simplify our approach to calculating signature scores to promote generalisability. We examined whether a
201 geometric mean calculation for the three component genes would perform as well as the original support vector
202 machine approach in our previous incipient[21] and presumptive TB[7] datasets. Since the simplified (geometric
203 mean) approach performed similarly, this method was used in all subsequent analyses (Supplementary Figure
204 8).

205 We quantified discrimination for each biomarker by constructing receiver operating characteristic (ROC) curves
206 and calculating areas under the curves (AUROCs) using the *pROC* package, with 95% confidence intervals
207 using the DeLong method[25]. We also compared AUROCs for each RNA signature to CRP using paired DeLong
208 tests, with adjustment for multiple testing using a Benjamini-Hochberg correction[25]. Sensitivities, specificities
209 and predictive values for each RNA signature were calculated using pre-specified cut-offs of two standard
210 scores above the mean of the healthy control population (Z2).

211 Subgroup analyses were performed by stratifying by W4SS status (presence of any of fever, cough, weight
212 loss or sweats) and CD4 count (<200 cells/mm^3 *vs.* ≥200 cells/mm^3). We also compared discrimination
213 between participants with a recorded TB diagnosis or treatment within six months of enrolment and those who
214 remained TB-free for this period, stratified by sputum culture, to assess if accuracy varied according to sputum
215 culture status. We compared discrimination for each signature between subgroups using unpaired Delong
216 tests, with adjustment for multiple testing as before.

217 Correlation between RNA signatures, CRP and physiological indices of disease severity were examined using
218 scatterplots and Spearman rank correlation coefficients. We also examined factors associated with higher RNA
219 signature scores using multivariable linear regression, with restricted cubic splines for continuous variables to
220 account for potential non-linear associations.

221 In order to investigate the clinical utility of candidate TB screening strategies, decision curve analysis was
222 performed using the *rmda* package[26], as described previously[27]. Briefly, decision curve analysis determines

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223 the 'net benefit' of diagnostic approaches, in comparison to intervening for all or no participants. Net benefit
224 reflects the true positive rate minus false positive rate weighted by the cost-benefit ratio across a range of
225 threshold probabilities which will trigger a decision, as a surrogate measure of the range of cost-benefit ratios.
226 The "intervention" in the context of a TB screening test is the offer of confirmatory testing (for example using
227 sputum culture), and the threshold probabilities reflect the minimum probability of disease at which further
228 investigation would be triggered. The ideal approach has the highest net-benefit across a clinically relevant
229 threshold probability range. The net-benefit of using the best performing RNA biomarker (at a threshold of Z2)
230 to guide confirmatory testing was assessed, compared to alternative strategies of confirmatory testing for all,
231 confirmatory testing for none, and confirmatory testing guided by CRP (cut-off ≥5mg/L as recommended in
232 WHO guidance[3,4]) or W4SS.

233 We also performed a range of exploratory analyses. First, we examined whether an optimised HIV-TB RNA
234 signature could be derived by temporally splitting the cohort into development (75%) and validation (25%) sets.
235 We then ranked the 23 measured transcripts by AUROC for TB culture status in the development set and
236 examined whether iteratively adding genes improved discrimination in the held-out validation set. We used a
237 range of approaches to combine individual genes to calculate overall scores, including simple calculations
238 (geometric means or disease risk scores[28]), and multivariable models trained on the development set (logistic
239 regression and support vector machines). Second, we used the same development/validation split to assess
240 whether a multivariable model including the most discriminating RNA signature, CRP and clinical predictors
241 (number of W4SS symptoms, haemoglobin, CD4 count and body mass index) may further improve
242 performance. For this, we used a multivariable logistic regression approach with restricted cubic splines to
243 model potential non-linear associations. Finally, we examined whether an approach combining CRP (≥5mg/L)
244 *and* the most discriminating RNA biomarker (Z-score ≥2) may offer better net benefit to guide confirmatory
245 testing in decision curve analysis.

246 **Sensitivity analyses**

247 We explored the effect of alternative reference standard definitions, as described above, in sensitivity analyses.
248 We also examined an alternative approach to Nanostring data batch-correction, by normalising probe-level
249 data to the mean of the reference RNA samples for each batch.

250 **Role of the funding source**

251 The funder had no role in study design, data collection, data analysis, data interpretation, writing of the report,
252 or decision to submit for publication. The corresponding authors had full access to all the data in the study and
253 had final responsibility for the decision to submit for publication.

254 **Results**

255 **Overview of study cohort**

256 A total of 862 participants were recruited to the parent study during the study period. Of these, 707 (82%) had
257 blood RNA and CRP data available and were included in the analysis (Figure 1, Table 1). There were no
258 systematic differences between included and excluded participants (Supplementary Table 2). Of the included
259 study population (n=707), median age was 32 years (interquartile range [IQR] 27-39), 407 (56%) were female
260 and median CD4 cell count was 306 cells/mm^3 (IQR 184-486). A total of 406 (57%) presented with at least

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261 one of the symptoms comprising the W4SS, while 633 (90%) of the included cohort had two sputum culture
262 results available. Of 676 participants with at least one sputum culture result, 89 (13.2%) were positive, while
263 65/699 (9.3%) with available Ultra results were positive. 130/707 (18%) of participants had a recorded TB
264 diagnosis or treatment within 6 months of enrolment; 84% and 89% of these cases were within 4 and 8 weeks,
265 respectively (Supplementary Figure 9). A total of 11/107 (10.2%) of those with known site of disease were
266 extra-pulmonary.

267 **Diagnostic accuracy of RNA signatures for culture-positive TB**

268 The seven RNA biomarkers had similar discrimination for sputum culture status, with AUROCs ranging from
269 0.73 for Zak11 (95% CI 0.68-0.79) to 0.80 for Suliman4 (95% CI 0.75-0.86) (Figure 2). All of the RNA
270 biomarkers had statistically equivalent discrimination to CRP in pairwise tests (CRP AUROC 0.78; 95% CI
271 0.72-0.83; Table 2). Using Z2 cut-offs, none of the signatures met the WHO-recommended minimum sensitivity
272 of 90% and specificity of 70% for a triage test (Table 2). Suliman4, the RNA signature with the highest AUROC
273 point estimate, had sensitivity and specificity of 0.83 (0.74-0.9) and 0.59 (0.55-0.63), respectively. Most
274 signatures had higher sensitivity than specificity at Z2 cut-offs, with 28-74% of participants having a score
275 above the Z2 threshold. Positive predictive values ranged from 16-27%, with negative predictive values 92-
276 97%. By comparison, CRP had sensitivity of 0.85 (0.77-0.91) and specificity 0.48 (0.44-0.52) at the primary
277 cut-off of ≥5mg/L, with 56% of participants having a positive result. For Suliman4 at Z2 threshold, the numbers
278 needed to test with confirmatory testing were 4.3 (3.5-5.3) and 23.9 (14.8-39.2) among Suliman4-positive and
279 negative participants. For CRP, numbers needed to test were similar at 5 (4.1-6.2) and 22.7 (13.5-38.6) among
280 CRP-positive and negative individuals, respectively.

281 In subgroup analyses, RNA signatures and CRP were less discriminating among W4SS-negative (AUROCs
282 0.56-0.65), compared to W4SS-positive participants (AUROCs 0.75-0.84; Table 3). There were no differences
283 in discrimination when stratified by CD4 count or sputum culture status of TB cases.

284 **Associations between RNA scores, indices of HIV/TB severity and CRP**

285 The seven RNA biomarkers were moderately to highly correlated (Spearman rank coefficients 0.42-0.93;
286 Supplementary Figure 10). Correlation was also observed between CRP and RNA biomarkers (Spearman
287 rank coefficients 0.23-0.61), though this tended to be weaker than that observed between the RNA biomarkers.

288 To examine whether RNA signature scores were associated with HIV/TB severity, we plotted scatterplots for
289 Suliman4 (the signature with the highest AUROC point estimate) with clinical measures of disease severity,
290 stratified by TB status (Supplementary Figure 11). Higher Suliman4 scores were associated with lower BMI,
291 CD4 count, haemoglobin and middle upper arm circumference, along with higher TBScoreII[29] and respiratory
292 rate among participants with and without TB (Supplementary Table 2). Among those with TB, higher Suliman4
293 scores were associated with higher smear grade and lower time to positivity in liquid culture. In multivariable
294 linear regression, number of symptoms, BMI, CD4 count, haemoglobin, respiratory rate and sputum culture
295 status were independently associated with higher Suliman4 scores (Supplementary Figure 12).

296 **Clinical utility**

297 In decision curve analysis, Suliman4 with a Z2 cut-off to guide confirmatory testing had higher net benefit than
298 an approach of confirmatory testing for all when the threshold probability exceeded ~4% (equivalent to a

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299 number willing to test with confirmatory testing of up to ~24 people per true TB case detected; Figure 3;
300 Supplementary Table 3). Using CRP ≥5mg/L had similar, albeit slightly lower net benefit to Suliman4. There
301 was a larger incremental net benefit for Suliman4 with increasing threshold probabilities (i.e. when the number
302 willing to test is lower). Both Suliman4 and CRP had higher net benefit than W4SS, which itself surpassed a
303 confirmatory testing for all approach above threshold probabilities of ~6% (equivalent to a number willing to
304 test with confirmatory testing of up to ~15 people per true TB case detected).

305 **Exploratory analyses**

306 Our forward search to identify an optimised RNA signature for HIV-TB did not lead to significantly improved
307 discrimination for TB culture status in the temporal validation set, when using simple calculations or
308 multivariable models to combine individual gene expression values (Supplementary Figure 13, Supplementary
309 Table 4). A multivariable model trained on the development set incorporating Suliman4, CRP and clinical
310 predictors (number of W4SS symptoms, haemoglobin, CD4 count and body mass index) also did not lead to
311 significant improvement in discrimination, with AUROC 0.81 (0.71 - 0.91) in the validation set. Our exploratory
312 approach of combining CRP (≥5mg/L) and Suliman4 (≥Z2) had sensitivity of 0.80 (0.70 - 0.87) and specificity
313 of 0.70 (0.66 - 0.74). In decision curve analyses, this approach had slightly higher net benefit than Suliman4
314 alone, with an incrementally greater net benefit at higher threshold probabilities (Figure 5).

315 **Sensitivity analyses**

316 Our sensitivity analyses using alternative reference standard definitions for TB and an alternative approach to
317 Nanostring data batch-correction did not lead to any substantial difference in the main results (Supplementary
318 Figures 15-17).

319 **Discussion**

320 To our knowledge, this is the first study to examine the diagnostic accuracy and clinical utility of a range of
321 promising blood RNA signatures for the application of systematic HIV-TB screening prior to ART initiation. We
322 found that the seven candidate signatures had similar diagnostic accuracy for culture-positive TB and were
323 moderately to highly correlated, supporting previous analyses. However, none of the candidate signatures met
324 the WHO target product profile criteria for a triage test and performance did not exceed that of CRP. The RNA
325 signature with the highest point estimate was Suliman4; both Suliman4 and CRP had superior clinical utility to
326 guide confirmatory testing compared to W4SS. Signature accuracy appeared independent of CD4 count and
327 the sputum culture status of TB cases. However, accuracy was lower among W4SS-negative participants for
328 all signatures and CRP, suggesting inferior performance in the absence of symptomatic disease.

329 While most RNA signatures either met or approached the WHO triage test sensitivity target of 90% at the Z2
330 cut-off, specificity was generally below the 70% target. This lack of specificity may reflect upregulation of
331 interferon activity that we have previously shown underpins expression of TB biomarker genes[6]. Such
332 upregulation of interferon activity may be driven by untreated HIV itself[12,14,15], and/or other opportunistic
333 infections. Notably, higher RNA signature scores were associated with indices of HIV severity (including lower
334 BMI, haemoglobin and CD4 count) in univariable and multivariable analyses, further supporting this
335 hypothesis. Collectively, these findings highlight the need to develop interferon-independent host response
336 biomarkers for TB screening among PLHIV.

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337 In our clinical utility analyses, we showed that both Suliman4 and CRP had higher net benefit than confirmatory
338 testing for all if the health service is willing to perform up to approximately 22 confirmatory tests per true TB
339 case diagnosed. If the health service can perform more confirmatory tests than this, then our analysis suggests
340 a confirmatory testing for all approach may be preferable. Of note, our exploratory approach combining CRP
341 (≥5mg/L) and Suliman4 (≥Z2) showed higher net benefit than either biomarker alone, with largely preserved
342 sensitivity of 80% and improved specificity of 70%. This finding reflects that blood RNA biomarkers and CRP
343 had weak to moderate correlation, they may therefore provide orthogonal information. Combining both has the
344 potential to improve specificity, however such an approach would incur additional cost, making it highly unlikely
345 to achieve the WHO target price for a triage test of <$2. Since CRP testing is already widely available, including
346 low-cost point-of-care platforms, our data support the programmatic roll-out of CRP for pre-ART TB screening[3],
347 while better biomarkers are sought.

348 Our study has numerous strengths, including the size of the cohort, with a representative sample of >700
349 participants newly referred to initiate ART. The cohort were well-characterised and intensively investigated for
350 TB, with 90% having two sputum culture results available, thus enabling a robust culture-based primary
351 reference standard, complemented by sputum induction when required. In addition, sputum Ultra and urine
352 diagnostics were systematically applied, and data linkage was performed to routinely collected data warehouse
353 records to identify participants who were diagnosed and treated for TB following study enrolment. This enabled
354 us to conduct secondary analyses using alternative reference standards, which supported the robustness of
355 our primary findings. We implemented a laboratory and analysis pipeline using the Nanostring platform to
356 measure seven candidate RNA signatures that have performed well in previous analyses[6–8]. The signatures
357 were curated through our previous systematic review[6] and were reproduced according to the original authors'
358 descriptions. The Nanostring pipeline demonstrated high levels of reproducibility and our head-to-head
359 analysis showed superior discrimination for TB status when compared to RNA sequencing data in a subset of
360 samples from our previously published presumptive TB cohort, thus reinforcing its robustness. Finally, CRP
361 was also measured, enabling comparative head-to-head analyses with the candidate RNA biomarkers.

362 Our study is limited to a single centre, precluding assessments of generalisability across settings. This setting
363 may be considered to be generally representative of populations with hyperendemic transmission of TB and
364 HIV, but the relatively low specificity of the biomarkers means that the positive predictive value of these tests
365 will reduce among PLHIV with lower prior probability of TB. Second, our targeted approach to RNA
366 quantification precludes development of novel signatures beyond the 23 measured transcripts. Further
367 genome-wide discovery will be required in such cohorts to identify novel biomarkers. Third, viral load was not
368 available. We were therefore unable to test the hypothesis that high viral loads are independently associated
369 with higher RNA signature scores. This limitation was mitigated by the availability of multiple other indices of
370 HIV severity, which were associated with RNA signature scores, but failed to improve the performance of
371 biomarkers in multivariable models.

372 In conclusion, RNA biomarkers showed better clinical utility to guide confirmatory TB testing for pre-ART
373 screening than W4SS, but their performance did not exceed that of CRP, and fell short of WHO mandated
374 targets. Interferon-independent approaches for host-response TB diagnostic screening may be required to
375 improve specificity among PLHIV prior to ART initiation. Until then, the clinical and health-economic impact of
376 widely available point-of-care CRP tests should be further evaluated for pre-ART TB screening.

377

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378 **Footnotes**

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381 and treatment data for patients for the period after study visit.

382 **Author contributions**

383 Conceived and designed the study: RKG, GT, MN

384 Sample and clinical data collection: BR, GN, HM, BD, HT, ZP, SN, DM

385 Laboratory analysis: TM, AC, PK

386 Data analysis: TM, BR, RKG, CJC, AC, PK, GT, MN

387 Manuscript preparation: TM, RKG, BR, GT, MN with input from all authors

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395 **Declaration of interests**

396 MN has a patent application pending in relation to blood transcriptomic biomarkers of tuberculosis. All other
397 authors declare no competing interests.

398 **Data availability statement**

399 Processed Nanostring data will be provided as a supplementary file, along with accompanying metadata at
400 the time of peer-reviewed publication.

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470 ## Tables

471 **Table 1**

472 ***Baseline characteristics of the included study cohort, stratified by culture status.***

Characteristic	Overall, N = 707	Negative, N = 587[1]	Positive, N = 89[1]	(Missing), N = 31[1]
Age (years)	32 (27, 39)	32 (26, 39)	35 (29, 43)	33 (26, 39)
Gender				
Female	407 (58%)	356 (61%)	35 (39%)	16 (52%)
Male	299 (42%)	230 (39%)	54 (61%)	15 (48%)
Missing	1	1	0	0
Previous TB	98 (14%)	82 (14%)	13 (15%)	3 (9.7%)
CD4 (cells/mm3)	306 (184, 486)	333 (206, 511)	204 (66, 314)	262 (81, 478)
Missing	5	2	2	1
CD4 <200 cells/mm3	193 (27%)	139 (24%)	42 (48%)	12 (40%)
Missing	5	2	2	1
Haemoglobin (g/dl)	12.70 (11.30, 13.90)	12.95 (11.50, 14.10)	11.35 (9.80, 12.57)	12.60 (11.55, 13.75)
Missing	150	127	19	4
Body mass index (kg/m2)	24 (21, 29)	25 (21, 30)	21 (19, 23)	24 (22, 27)
Missing	2	1	1	0
Middle upper arm circumference (cm)	27.0 (25.0, 30.0)	28.0 (25.0, 31.0)	25.0 (23.0, 27.0)	27.0 (25.0, 29.2)
WHO 4-symptom screen positive	406 (57%)	311 (53%)	70 (79%)	25 (81%)
TBscoreII	1.00 (0.00, 2.00)	1.00 (0.00, 1.00)	2.00 (1.00, 3.00)	1.00 (0.00, 2.00)
Missing	36	26	9	1
CRP (mg/L)	6 (2, 32)	5 (2, 19)	71 (11, 146)	10 (3, 52)
Number of valid sputum cultures				
0	31 (4.4%)	0 (0%)	0 (0%)	31 (100%)
1	43 (6.1%)	39 (6.6%)	4 (4.5%)	0 (0%)
2	633 (90%)	548 (93%)	85 (96%)	0 (0%)
Sputum smear	21 (3.0%)	7 (1.2%)	13 (15%)	1 (5.6%)
Missing	13	0	0	13
Sputum Ultra				
Negative	616 (88%)	572 (98%)	23 (26%)	21 (88%)

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Characteristic	Overall, N = 707	Negative, N = 587[1]	Positive, N = 89[1]	(Missing), N = 31[1]
Trace	18 (2.6%)	10 (1.7%)	6 (6.7%)	2 (8.3%)
Positive	65 (9.3%)	4 (0.7%)	60 (67%)	1 (4.2%)
Missing	8	1	0	7
Urine LAM	18 (2.6%)	6 (1.0%)	10 (11%)	2 (6.5%)
Missing	3	3	0	0
Urine Ultra	37 (5.3%)	11 (1.9%)	24 (27%)	2 (6.5%)
Missing	6	5	1	0
Sputum culture or Ultra positive	94 (13%)	4 (0.7%)	89 (100%)	1 (4.5%)
Missing	9	0	0	9
Any positive TB test	112 (16%)	21 (3.6%)	89 (100%)	2 (8.7%)
Missing	8	0	0	8
Recorded TB diagnosis or treatment within 6 months	130 (18%)	37 (6.3%)	85 (96%)	8 (26%)
TB site				
Extra Pulmonary TB	11 (10%)	9 (29%)	1 (1.4%)	1 (14%)
Pulmonary TB	96 (90%)	22 (71%)	68 (99%)	6 (86%)
Missing	600	556	20	24

[1]Statistics presented: median (IQR); n (%)

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475 **Table 2**

476 ***Blood RNA biomarker and CRP performance metrics for discrimination of sputum culture status.***

477 P values indicate pairwise comparisons to CRP, with multiple testing correction (n = 676 participants).

Signature	AUROC	Sensitivity	Specificity	PPV	NPV	Triage positive	NNT(+)	NNT(-)	p
Suliman4	0.8 (0.75 - 0.86)	0.83 (0.74 - 0.9)	0.59 (0.55 - 0.63)	0.23 (0.19 - 0.28)	0.96 (0.93 - 0.97)	0.47 (0.43 - 0.51)	4.3 (3.5 - 5.3)	23.9 (14.8 - 39.2)	0.320
Risk6	0.79 (0.74 - 0.85)	0.91 (0.83 - 0.95)	0.39 (0.35 - 0.43)	0.18 (0.15 - 0.22)	0.97 (0.94 - 0.98)	0.65 (0.61 - 0.68)	5.4 (4.5 - 6.6)	29.8 (15.4 - 58.4)	0.575
Sweeney3	0.79 (0.73 - 0.85)	0.92 (0.85 - 0.96)	0.28 (0.25 - 0.32)	0.16 (0.13 - 0.2)	0.96 (0.92 - 0.98)	0.74 (0.71 - 0.77)	6.1 (5 - 7.5)	24.9 (12.4 - 51)	0.608
Gliddon3	0.79 (0.73 - 0.84)	0.89 (0.81 - 0.94)	0.31 (0.28 - 0.35)	0.16 (0.13 - 0.2)	0.95 (0.91 - 0.97)	0.71 (0.68 - 0.75)	6.1 (5 - 7.5)	19.4 (10.8 - 35.4)	0.608
CRP	0.78 (0.72 - 0.83)	0.85 (0.77 - 0.91)	0.48 (0.44 - 0.52)	0.2 (0.16 - 0.24)	0.96 (0.93 - 0.97)	0.56 (0.53 - 0.6)	5 (4.1 - 6.2)	22.7 (13.5 - 38.6)	NA
BATF2	0.75 (0.69 - 0.8)	0.82 (0.73 - 0.89)	0.53 (0.49 - 0.57)	0.21 (0.17 - 0.25)	0.95 (0.92 - 0.97)	0.52 (0.48 - 0.56)	4.8 (3.9 - 5.9)	20.4 (12.8 - 32.9)	0.405
Roe3	0.74 (0.68 - 0.8)	0.73 (0.63 - 0.81)	0.61 (0.57 - 0.65)	0.22 (0.18 - 0.27)	0.94 (0.91 - 0.96)	0.44 (0.4 - 0.48)	4.6 (3.7 - 5.7)	15.8 (10.8 - 23.4)	0.320
Zak11	0.73 (0.68 - 0.79)	0.56 (0.46 - 0.66)	0.77 (0.73 - 0.8)	0.27 (0.21 - 0.34)	0.92 (0.89 - 0.94)	0.28 (0.24 - 0.31)	3.7 (3 - 4.8)	12.6 (9.3 - 17)	0.320
W4SS	0.63 (0.58 - 0.68)	0.79 (0.69 - 0.86)	0.47 (0.43 - 0.51)	0.18 (0.15 - 0.23)	0.94 (0.9 - 0.96)	0.56 (0.53 - 0.6)	5.4 (4.4 - 6.8)	15.5 (10.2 - 24)	0.000

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479 **Table 3.**

480 ***Blood RNA biomarker and CRP performance metrics for discrimination of TB status, stratified by***
481 ***selected subgroups.***

482 Accuracy shown for primary outcome of sputum culture status as area under the receiver operating
483 characteristic curve with 95% confidence intervals. Reference standard for W4SS and CD4 strata is sputum
484 culture, as per primary reference standard. Reference standard for the sputum culture strata is based on TB
485 diagnosis or treatment recorded within six months of enrolment. P values indicate comparisons between strata
486 for each signature.

Signature	W4SS-positive	W4SS-negative	p
BATF2	0.78 (0.72 - 0.84)	0.59 (0.43 - 0.74)	0.029
CRP	0.81 (0.76 - 0.87)	0.56 (0.42 - 0.7)	0.003
Gliddon3	0.83 (0.77 - 0.89)	0.58 (0.44 - 0.72)	0.003
Risk6	0.83 (0.78 - 0.89)	0.59 (0.46 - 0.72)	0.003
Roe3	0.77 (0.71 - 0.83)	0.57 (0.41 - 0.74)	0.029
Suliman4	0.84 (0.79 - 0.89)	0.6 (0.46 - 0.74)	0.003
Sweeney3	0.84 (0.79 - 0.9)	0.56 (0.42 - 0.7)	0.002
Zak11	0.75 (0.68 - 0.81)	0.65 (0.51 - 0.78)	0.193
Signature	CD4 <200 cells	CD4 >=200 cells	p
BATF2	0.76 (0.68 - 0.84)	0.7 (0.62 - 0.79)	0.482
CRP	0.84 (0.77 - 0.91)	0.7 (0.61 - 0.78)	0.112
Gliddon3	0.83 (0.75 - 0.9)	0.72 (0.63 - 0.81)	0.188
Risk6	0.83 (0.75 - 0.9)	0.73 (0.65 - 0.82)	0.188
Roe3	0.75 (0.66 - 0.83)	0.7 (0.61 - 0.79)	0.512
Suliman4	0.84 (0.76 - 0.92)	0.75 (0.67 - 0.83)	0.188
Sweeney3	0.84 (0.76 - 0.91)	0.73 (0.65 - 0.82)	0.188
Zak11	0.71 (0.62 - 0.8)	0.74 (0.65 - 0.82)	0.667
Signature	Culture-positive TB	Culture-negative TB	p
BATF2	0.75 (0.7 - 0.81)	0.69 (0.61 - 0.77)	0.447
CRP	0.79 (0.73 - 0.85)	0.79 (0.72 - 0.86)	0.978
Gliddon3	0.8 (0.74 - 0.86)	0.76 (0.68 - 0.84)	0.549
Risk6	0.8 (0.75 - 0.86)	0.75 (0.67 - 0.83)	0.447
Roe3	0.75 (0.69 - 0.81)	0.69 (0.61 - 0.77)	0.447
Suliman4	0.82 (0.76 - 0.87)	0.79 (0.72 - 0.86)	0.689
Sweeney3	0.8 (0.74 - 0.85)	0.7 (0.61 - 0.78)	0.361
Zak11	0.74 (0.69 - 0.8)	0.67 (0.6 - 0.75)	0.447

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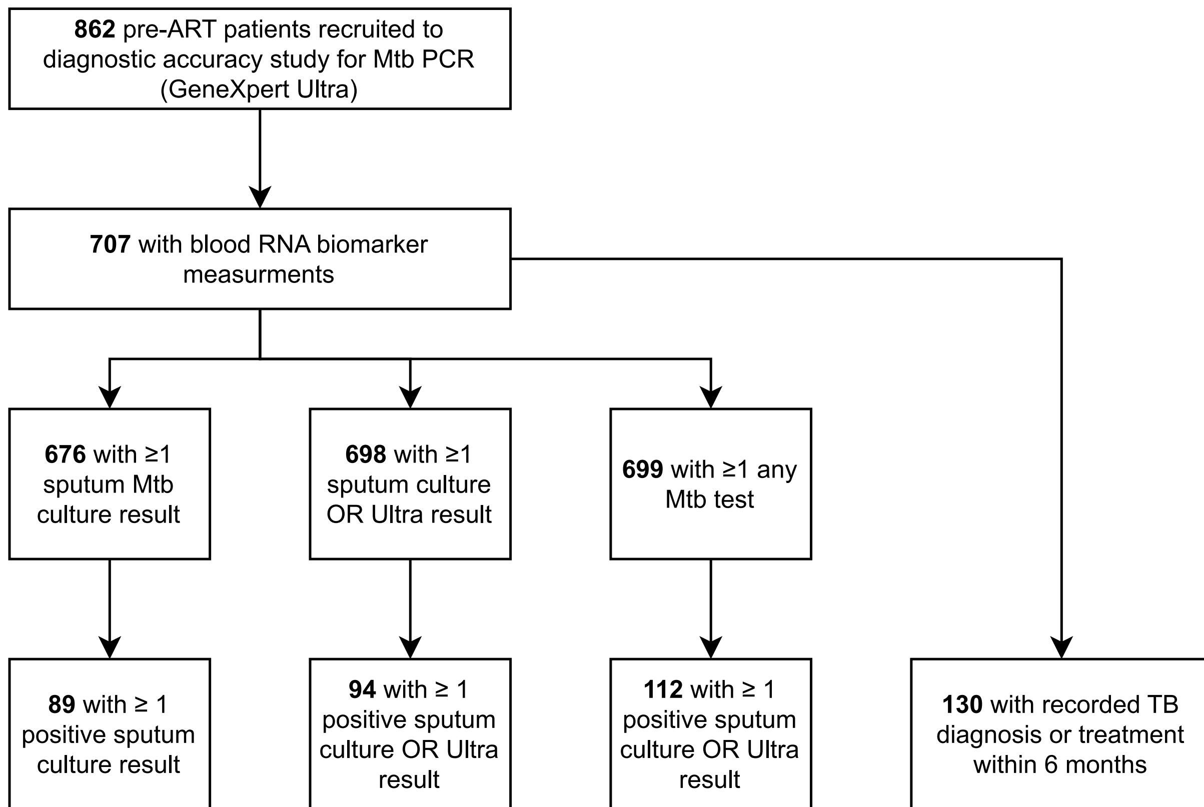
488 ## Figures

489 **Figure 1**

490 ***Consort diagram.***



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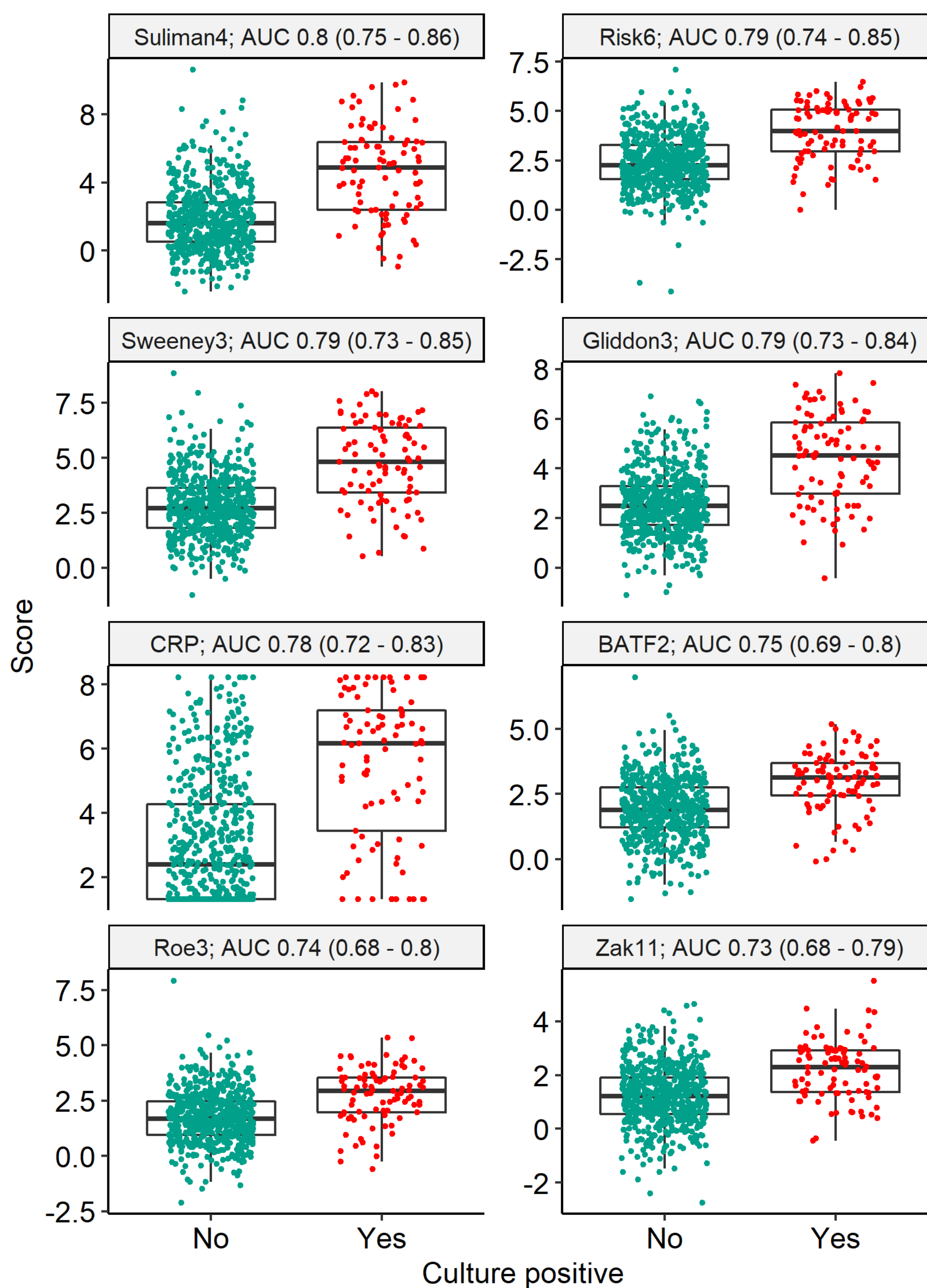
493 **Figure 2**

494 ***Blood RNA biomarker discrimination of Mtb sputum culture positivity***

495 Scores and discrimination of RNA signatures and CRP for primary outcome of sputum culture positivity (n =
496 676 participants). Scores are shown as Z-scores for RNA signatures, and log-2 transformed CRP (mg/L).
497 Discrimination presented as area under the receiver operating characteristic curve (AUC), with 95% confidence
498 intervals.



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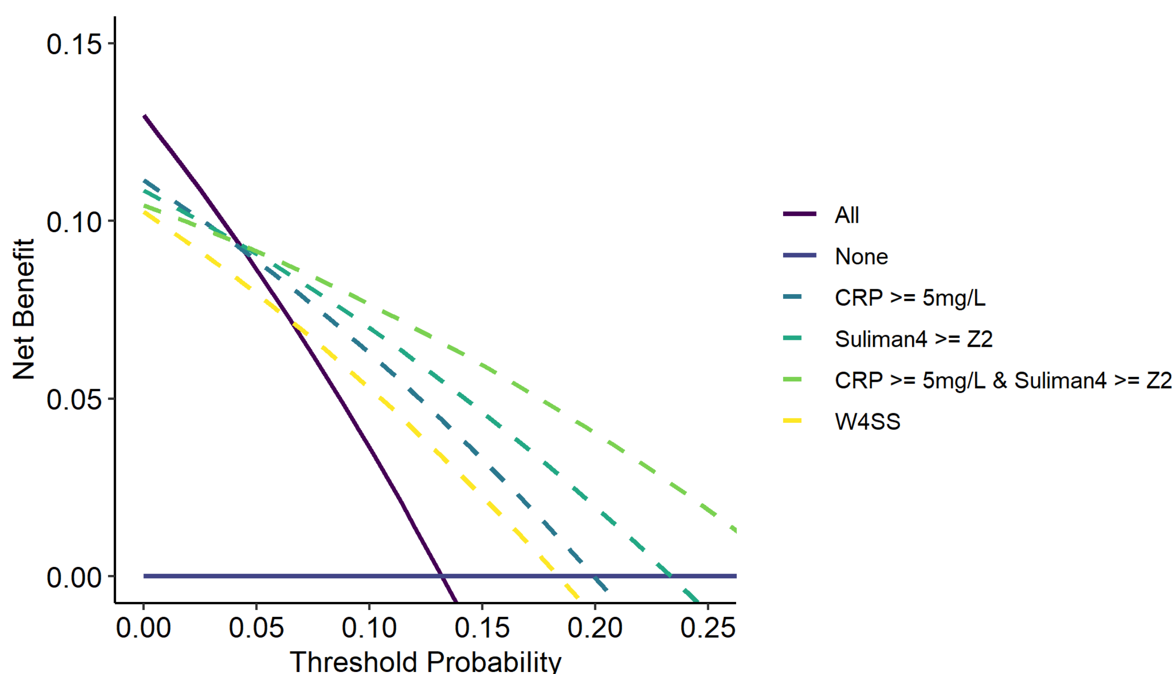
500 **Figure 3**

501 ***Decision curve analysis for alternative triage strategies to trigger confirmatory investigations for TB***

502 Net benefit (true positive rate - false positive rate weighted by cost:benefit ratio) for investigate all and
503 investigate none approaches across the range of threshold probabilities that a service may use to trigger
504 confirmatory investigations for TB is compared to that of decisions to investigate triggered by each of triage
505 strategies indicated: CRP (≥5 and ≥10mg/L), Suliman4 (Z2), symptoms (W4SS) and using an exploratory
506 approach of CRP>=5 and Suliman4 >=Z2.



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