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Title: Four-gene pan-African blood signature predicts progression to tuberculosis

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All authors reviewed, provided feedback and approved the manuscript and are accountable for the accuracy and integrity of the work

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At a glance commentary

Intervention against the tuberculosis (TB) epidemic requires a multi-pronged approach, including treatment and prevention. TB exists in a dynamic spectrum from latent infection to disease, and only about 5 to 10% of infected individuals develop clinical TB. Therefore, the reservoir for TB is huge since 1.7 billion people globally are estimated to be infected with the causative pathogen:

Mycobacterium tuberculosis (*M.tb*). Consequently, identifying asymptomatic individuals who are at high risk of progressing to TB would help prioritize preventative strategies, which would provide an important step forward towards better TB control. We developed a blood test to predict progression towards active TB in multiple HIV-negative Sub-Saharan African populations, following exposure to an index (active) TB patient living in the same household. The test statistically predicted TB progression in different African cohorts. This simple 4-marker test could be translated into a simple, rapid and affordable point-of-care test for field application in resource-limited settings, where TB and *M.tb* infection are endemic, to identify individuals at high risk of developing TB. High-risk TB contacts could then be prioritized for prophylactic interventions.

Online data supplement: This article has an online data supplement, which is accessible from this issue's table of content online at www.atsjournals.org

Abstract

Rationale: Contacts of tuberculosis (TB) patients constitute an important target population for preventative measures as they are at high risk of infection with *Mycobacterium tuberculosis* and progression to disease.

Objectives: We investigated biosignatures with predictive ability for incident tuberculosis.

Methods: In a case-control study nested within the Grand Challenges 6-74 longitudinal HIV-negative African cohort of exposed household contacts, we employed RNA sequencing, polymerase chain reaction (PCR) and the Pair Ratio algorithm in a training/test set approach. Overall, 79 progressors, who developed tuberculosis between 3 and 24 months following exposure, and 328 matched non-progressors, who remained healthy during 24 months of follow-up, were investigated.

M*easurements and Main Results*: A four-transcript signature (RISK4), derived from samples in a South African and Gambian training set, predicted progression up to two years before onset of disease in blinded test set samples from South Africa, The Gambia and Ethiopia with little population-associated variability and also validated on an external cohort of South African adolescents with latent *Mycobacterium tuberculosis* infection. By contrast, published diagnostic or prognostic tuberculosis signatures predicted on samples from some but not all 3 countries, indicating site-specific variability.

Post-hoc meta-analysis identified a single gene pair, C1QC/TRAV27, that would consistently predict TB progression in household contacts from multiple

African sites but not in infected adolescents without known recent exposure events.

Conclusions: Collectively, we developed a simple whole blood-based PCR test to predict tuberculosis in household contacts from diverse African populations, with potential for implementation in national TB contact investigation programs.

Abstract word count: 246

MeSH key words: tuberculosis, gene expression, biomarkers

Introduction

Tuberculosis (TB), caused by infection with *Mycobacterium tuberculosis* (*M.tb*)[1, 2], is the leading cause of death caused by a single pathogen globally[3]. Prior to development of symptomatic disease, latent *M.tb* infection can be detected by measuring immunological sensitization, using the tuberculin skin test (TST) and/or interferon gamma release assays (IGRA)[4]. Most infected individuals have effective defense mechanisms to control *M.tb*[5] as only 5-10% will progress to TB during their lifetime. Despite this, over 10 million new cases of TB are diagnosed each year from either *M.tb* reactivation or direct transmission, and almost 2 million people die from the disease[3]. Although recent *M.tb* exposure and TST or IGRA conversion are associated with higher risk of TB progression[6], the positive predictive values of these tests are low, i.e. 1.5% and 2.7%[7], falling short of current WHO supported guidelines for incipient TB[8]. Thus, the number of TST or IGRA-positive individuals requiring treatment to prevent progression to a single incident case of TB is prohibitively high[9].

Factors associated with elevated risk of progression to TB include age, sex, HIV [10, 11], and especially being in recent contact with a patient with active pulmonary TB[12, 13]. A biomarker that identifies household contacts (HHC) who will progress to TB would provide an opportunity to arrest disease progression through targeted prophylactic intervention[14, 15]. Such prognostic biomarkers would be most impactful as point-of-care tests for resource-limited settings, such as those in Sub-Saharan Africa. Test performance should not be adversely affected by diversity of ethnic backgrounds[16] and circulating *M.tb* lineages[17], as

seen in Africa. A ‘TB-risk’ test must be practical for field application and therefore based on accessible biological samples routinely used in clinical settings, such as peripheral blood[18].

Transcriptional profiling of blood cells has emerged as a powerful platform to discover potential TB biomarkers discriminating TB patients from healthy uninfected and/or latently *M.tb*-infected individuals[19, 20, 21, 22, 23, 24]. We previously defined a 16-gene blood transcriptional correlate of risk (COR) signature that predicts risk of progression to TB in *M.tb*-infected HIV-negative South African adolescents and HHC from South Africa and The Gambia[25]. However, given that this COR signature was developed using a single cohort of latently *M.tb*-infected South African adolescents, the predictive accuracy for HHC in diverse African populations may be sub-optimal[25]. It would also be desirable to reduce the number of transcripts in the signature, to facilitate implementation of a point-of-care test. These simple tests pave the way for simple identification of individuals at highest risk for progression. Some of the results of this study have been previously reported in the form of abstracts[26, 27].

Methods

Study design and participants

All clinical sites adhered to the Declaration of Helsinki and Good Clinical Practice guidelines. Ethical approvals were obtained from institutional review boards **(Supplementary Table 1, and online supplement)**. The HHC study included participants from four African sites: South Africa, The Gambia, Ethiopia and Uganda, under the Bill and Melinda Gates Grand Challenges 6-74 (GC6-74) program **(Figure 1 and Supplementary Table 2)**. Samples were collected at enrolment/baseline, 6 and 18 months, with the exception of South Africa, where samples at 6 months were not available. The Adolescent Cohort Study was described previously[25, 28] and included IGRA+ and/or TST+ South African adolescents aged 12-18 years old with *M.tb* infection, occurring at unspecified times. Adult participants, or legal guardians of participants aged 10-17 years old, provided written or thumb-printed informed consent to participate after careful explanation of the study and potential risks.

Sample processing and RNA-sequencing

PAXgene (PreAnalytiX, Hombrechtikon, Switzerland) blood RNA samples were collected from all participants. Progressors were defined as individuals who developed TB 3-24 months post-HHC. Non-progressor samples were matched to the pre-diagnosis time points of each progressor by site, gender, age and recruitment year (**online supplement**). RNA-sequencing was performed by Beijing Genomics Institute (Shenzhen, China); additional details for processing

and quality control are provided in the online supplement. FASTQ files have been deposited into the Gene Expression Omnibus under accession GSE94438.

Identification of predictive signatures

The hypothesis of the study was that gene expression signatures exist that can predict incident TB in recent household contacts of active TB patients.

Candidate site-specific signatures of risk for TB disease progression and final, simplified qRT-PCR-based candidate signatures were developed using the *Pair Ratios algorithm* (**online supplement**), which was previously described[29] and is a variation of the pairwise approach used to discover the ACS COR signature[25]. To summarize, the step-by-step procedure for computing the RISK4 signature scores using sample qRT-PCR measurements was:

1. Measure the cycle thresholds (Cts) for the four primer-probes (Applied Biosystems TaqMan Assays) listed in **Supplementary Table 3**.
2. For each of the four pairs of primer-probes, compute the difference in raw Ct, which produces the log-transformed ratio of expression.
3. Compare the measured ratio to ratios in the look-up table for the given pair of transcripts in **Supplementary Tables 4-7**. Find the minimal ratio in column 1 of the table that is greater than or equal to the measured ratio.
4. Assign the corresponding score in the second column of the look-up table to the ratio. If the measured ratio is larger than all ratios in column 1 of the look-up table, then assign a score of 1 to the ratio.

5. Compute the average over the scores generated from the set of pairs. If any assay failed on the sample, compute the average score over all ratios not including the failed assays. The resulting average is the final score for that sample.

Adaptation of published diagnostic signatures to qRT-PCR

The previously published signatures from Maertzdorf et al [30] and Sweeney et al [31] were adapted to the qRT-PCR platform, where we refer to them as DIAG4 and DIAG3, respectively. Primer-probe sets were selected for each gene in the respective signatures, and overall scores were computed for each sample as the difference in the mean of the up-regulated and the down-regulated transcripts (**Supplementary Tables 8-9).** All statistical analyses were performed in R (version 3.1.0) using the R package pROC[32]. Details are in the online supplement.

Results

We enrolled 4,466 HIV-negative healthy HHC of 1,098 index TB cases between 2006 and 2010 into the GC6-74 cohorts across 4 African sites **(Figure 1 and Supplementary Table 2)**. Samples from Uganda were not available in sufficient quantities for this analysis **(Figure 1)**. TB incidence in HIV-negative healthy HHC was highest in South Africa, and lowest in Ethiopia **(Table 1)**, as defined by TB case classifications A-K in **Supplementary Table 10**. Incident cases (progressors) were defined as those who developed TB between 3 and 24 months following exposure. "Co-incident" cases, i.e. diagnosed with TB within 3 months of contact with the index case **(Methods)**, were not included in analysis. Prior TB was an exclusion criterion **(online supplement)**, thus progressors likely had their first TB episode during follow-up. Median age of progressors was comparable across the 4 African sites (Kruskal-Wallis $p=0.92$, **Table 1**). Median times to progression were 7 months in South Africa and Uganda, and 10.5 and 10 months in The Gambia and Ethiopia, respectively **(Table 1, and Supplementary Table 11A)**. Progressors, as defined by clinical symptoms, chest and other radiographs (CXR) consistent with TB and response to chemotherapy, without microbiological confirmation comprised 25% (4/12) of progressors in Ethiopia, 2% (1/43) in South Africa and 6% (3/34) in The Gambia (TB classification K, **supplementary Tables 10 and 11A)**.

A four-gene correlate of risk signature predicts TB progression in household contacts

We divided South African and Gambian HHC cohorts into training and test sets, while the entire Ethiopian cohort was assigned to the test set due to its small sample size (**Figure 1, and Supplementary tables 11A and 11B**). We utilized the South African and Gambian training sets to construct site-specific signatures of TB risk, using RNA-seq transcriptomes and the Pair Ratio approach, which uses ratios of transcripts regulated in opposite directions during TB progression (**online supplement** and **Supplementary Tables 12 and 13**). Leave-one-out cross-validation analysis (LOOCV; applied to all samples from specific individuals) indicated strong potential for predicting TB progression in both cohorts (South Africa: **Figure 2A**; area under the receiver operating characteristic curve (AUC)=0.86 [95% CI: 0.79-0.94], $p=8.4x10^{-10}$; The Gambia: **Figure 2B;** AUC=0.77 [0.66-0.88]; $p=2.5x10^{-10}$). Applying the algorithm to the South African and Gambian cohorts generated two distinct site-specific risk signatures **(Figure 2C and D).** When measured by qRT-PCR using primer/probe sets that corresponded to the exons, predictive accuracy was maintained **(Supplementary Figure 1)**. The Gambia signature failed to validate on samples from South Africa (**Figure 2A**; AUC=0.59 [0.46-0.73], *p=0.061*), while the South Africa signature weakly validated on Gambian samples (**Figure 2B**; AUC=0.66 [0.54-0.76], $p=8.8X10^{-3}$), suggesting site-specific progression signatures in South Africa and The Gambia.

The poor cross-prediction of South Africa and The Gambia signatures motivated explicit development of a multi-cohort signature using a training set that combined samples from both sites. We pooled PCR-based transcript pairs

that comprised all the South Africa (38 transcripts), and The Gambia (35 transcripts) signatures (**Figure 2C and D, and Supplementary Tables 12 and 13)** to identify top transcript pairs that were significantly predictive of TB progression in both cohorts, and successively added the next best pair to the ensemble and re-assessed the predictive power at each stage until the gain in predictive performance reached a plateau **(Online supplement and Supplementary Table 14)**. This resulted in the RISK4 signature comprising four unique genes: GAS6 and SEPT4, which were up-regulated, whereas CD1C and BLK, which were down-regulated in progressors vs. matched controls **(Figure 3A)**.

Having developed a multi-site PCR-based signature of risk, we validated it by blind prediction of TB progression on the multi-cohort test sets from South Africa, The Gambia and Ethiopia **(Figure 1)**. The RISK4 signature significantly predicted progression in the entire combined test set (AUC=0.67 [0.57-0.77], $p=2.6X10^{-4}$, **Figure 3B**), and on each individual site (South Africa, The Gambia, and Ethiopia with AUCs: 0.66-0.72, p<0.03, **Figure 3B**). Surprisingly, performance of the signature on combined test set samples within a year of TB diagnosis (AUC=0.66 [0.55-0.78], $p=1.9X10^{-3}$, **Figure 3C**) was comparable to samples collected more than a year before diagnosis (AUCs=0.69 [0.51-0.86], *p=0.015*). Since deploying a risk signature in a screen-and-treat strategy in TB HHC would most likely entail testing early after exposure, we assessed the predictive performance of RISK4 on samples from HHC collected within two months of diagnosis of the index case. RISK4 also validated in this setting

(**Figure 3D**; AUC=0.69 [0.52-0.86], $p=4.8X10^{-3}$). Finally, to test robustness of RISK4, we performed blinded predictions on samples from an external cohort of IGRA+/TST+ South African adolescents (the "ACS" cohort), where the time of TB exposure was unknown[25]. RISK4 also significantly predicted risk of TB progression in this cohort (**Figure 3E**; AUC=0.69 [0.62-0.76], $p=3.4X10^{-7}$).

Comparison of RISK4 with published diagnostic TB signatures

To benchmark the predictive performance of the RISK4 signature, we compared it to qRT-PCR-based versions of three published transcriptional signatures for TB diagnosis: "DIAG3"; the 3-gene diagnostic signature by Sweeney et al[31], and "DIAG4"; the 4-gene diagnostic signature by Maertzdorf et al[30], and our own previously-reported 16-gene COR signature for TB progression ("ACS COR", Zak et al[25]). Since HHC training set samples were used to discover the RISK4 signature, we compared the performance of PCR-adapted published signatures to RISK4 in the HHC test set only. The three signatures predicted TB progression in the combined test set with comparable accuracy to RISK4 (**Figure 4A**, AUCs of 0.64-0.68, $p<3X10^{-3}$). When we compared the predictive accuracy of RISK4 to each of the 3 PCR-adapted signatures, the AUCs were not statistically different **(Supplementary Table 15)**. However, unlike RISK4 (**Figure 3B**), the three other signatures did not validate on all sites when evaluated individually (**Figures 4B-D**), suggesting that RISK4 represents a more generalizable prognostic signature.

After unblinding the South African, Gambian, and Ethiopian test sets, we interrogated whether the RISK4 signature could be reduced to a single pair of transcripts without a loss of predictive accuracy. We applied each of the four ratios in the RISK4 signature to each of the test set cohorts individually, and compared the performance to the entire RISK4 signature **(Supplementary Table 16)**. The ratio between the SEPT4 and BLK primers reproduced the performance of the RISK4 signature on all three test set cohorts, demonstrating feasibility of a highly simplified, 2-gene host RNA-based signature for identifying HHC at greatest risk of progressing to active TB.

Meta-analysis identifies gene pairs that predict TB progression across Africa

Overall, predictions for TB progression were the least accurate for the Ethiopian cohort, which was not used to develop the initial RISK4 signature **(Figures 1, 3 and 4)**. To determine whether further improved accuracy could be achieved for a signature performing well at all sites, we performed a meta-analysis of RNA-seq profiles for the combined training and test datasets from all our three cohorts. This post-hoc analysis was performed after unblinding of the test set, and was focused on identifying better predictive gene pairs, given that the single transcript pair SEPT4/BLK performed equivalently to the RISK4 signature (**Supplementary Table 16**).

We combined RNA-seq data from all training and test cohorts, thus merging the three independent cohorts from South Africa, The Gambia and

Ethiopia. Pairs of up-regulated and down-regulated transcripts were formed from all transcripts that individually discriminated progressors from controls in at least one cohort (**Supplementary Tables 17 and 18;** Wilcoxon FDR<0.05 in at least one of the three cohorts). Each pair was then analyzed on each of the three sites. We identified nine transcript pairs that discriminated progressors from controls with AUC>0.75 on all three sites (**Supplementary Table 19)**. The optimal pair consisted of C1QC (up-regulated) and TRAV27 (down-regulated) and achieved AUC>0.76 on all three sites. We performed logistic regression analysis to determine whether the remaining eight pairs **(Supplementary Table 20, Supplemental Methods)** captured information about TB progression that was redundant or complementary to the signals detected by C1QC/TRAV27. The ratio between ANKRD22 (up-regulated with TB progression) and OSBPL10 (down-regulated with progression) led to significantly increased discrimination between progressors and controls when it was combined with the C1QC/TRAV27 ratio in HHC cohorts **(Figures 5A-C)**, increasing the ROC AUC on all three HHC cohorts individually to AUC>0.79 **(Supplementary Table 21)**. Thus, the ratios C1QC/TRAV27 and ANRKD22/OSBPL10 capture distinct aspects of TB progression signals in HHC that are shared across three distinct African sites.

To determine whether the C1QC/TRAV27 and ANKRD22/OSBPL10 signatures captured universal aspects of TB progression rather than HHC-associated biology, we evaluated them using data from the cohort of IGRA+TST+ South African adolescents[25]. The ANKRD22/OBSPL10 ratio strongly predicted

TB progression among the *M.tb*-infected adolescents (**Figure 5D**; AUC=0.75 [0.68-0.81], $p=2.86x10^{-11}$), but the C1QC/TRAV27 ratio was poorly predictive in the adolescent cohort (**Figure 5D**; AUC=0.57 [0.49-0.64], p=0.042). In contrast to the HHC, combining the two ratios did not lead to improved discrimination of progressors and controls in the adolescent cohort (AUC=0.69 [0.61-0.76]; **Figure 5D and Supplementary Figure 2A**). To further understand the disparity in the predictive performance for the HHC cohorts and the *M.tb*-infected adolescents, we evaluated the longitudinal behavior of the transcript ratios for progressor samples in the HHC and adolescent cohorts (**Figures 5F and 5G**). The ANKRD22/OSBPL10 pair exhibited similar behavior in the HHC and ACS, with a steady up-regulation during progression and no significant difference between GC6-74 and adolescent participants in any 6-month time window preceding TB diagnosis **(Figure 5F)**. In contrast, the C1QC/TRAV27 ratio was significantly higher in HHC progressors than in *M.tb*-infected adolescents 19-24 months before TB diagnosis ($p=3X10^{-3}$, **Figure 5G)**. Importantly, samples from HHC progressors were collected mostly at enrolment, immediately following exposure to the respective TB index cases, thus possibly representing a signature of recent *M.tb* exposure.

Discussion

We identified and validated a simple, PCR-based transcriptomic signature, "RISK4", to predict risk of progression to active TB disease in diverse African cohorts of recently exposed HHC of index TB cases. This four-gene signature

predicted risk of progression with similar accuracy in 4 cohorts from 3 Sub-Saharan African populations with heterogeneous genetic backgrounds, TB epidemiology and circulating *M.tb* strains[33]. Importantly, RISK4 exhibited consistent predictive performance in all test set cohorts, while previously reported signatures[25, 30, 31] exhibited cohort-specific variability in performance. We previously reported that the ACS COR signature validated on the combined South African and Gambian HHC cohorts[25]. In the present analysis the stochastic partitioning of HHC samples into training and test sets results in different ACS COR performance measures to the previously reported results.

The signatures reported herein represent significant and translational improvements over currently used biomarkers for predicting risk of TB, such as IGRAs or TST[14, 15]. Recent estimates suggest the TB incidence of South Africa and The Gambia to be 0.8%[3] and 0.3%[34], respectively. However, IGRA and TST-positive prevalence can reach up to 50% in The Gambia and 80% in South Africa[3] and although IGRA and TST have a high (approximately 80%) sensitivity for *M.tb* infection, they have poor positive predictive values (PPV) of 2.7% and 1.5%, respectively for TB progression. Therefore, dozens of individuals would require prophylactic treatment to prevent progression to TB in a single individual[35, 36]. The WHO recently published guidelines for incipient TB target product profile to predict TB progression[8], to ensure that individuals at high risk of TB progression are not falsely excluded[7, 18], but are referred for additional investigation for TB or offered prophylactic treatment[37]. At sensitivities of 81, 71, 62 and 50% the RISK4 signature achieves specificities of 34, 52, 63 and 77% in

healthy asymptomatic individuals, respectively, by selection of different thresholds **(Supplementary Table 22).** Although RISK4 has a similar poor PPV of 3% to IGRA or TST, due to its lower sensitivity at higher specificity thresholds **(Supplementary Table 23)**, it importantly has lower positivity rates in the target population. To achieve a test performance similar to IGRAs (between 70 to 80% sensitivity and the number to harm (NTH) to prevent one case of approximately 85), the RISK4 threshold would identify between 38 and 54% of household contacts for preventative measures, compared to 78% for IGRA **(Supplementary Table 22)**.

There are several limitations in this study, including the small sample size (only 100 of the 4460 HHC progressed to active TB). Furthermore, although the intended application is for a trans-Africa test, we could only sample from three regions. The poor RISK4 performance in Ethiopia calls for larger multi-centered studies, particularly of under-represented populations throughout Africa, and other TB endemic areas. Although we defined 2-transcript signatures that have broader application, the sample size was not adequate to have unblinded validation sets, or to ensure that transcript pairs were not selected by chance during our post-hoc analyses. The test is based on blood samples, which are easily and routinely obtained in laboratory diagnostics. However, the test still requires translation into field-friendly instrumentation, to bypass the multi-step processing involved in RNA extraction and RT-PCR. Encouragingly, recent advances in point-of-care PCR technologies offer promise for developing rapid diagnostics. We envision platforms where blood from a finger prick can be

translated through field-friendly, handheld PCR instruments to interpretable scores. Field staff would then triage near-patient contacts into low-risk and higher-risk groups for further assessment and potential treatment for subclinical or active TB disease[8]. One advantage of the calculation of RISK4 or the 2-transcript scores is the pair-ratio structure, which eliminates the need for housekeepers or other standardization methods. As a proof of principle, a clinical trial stratifying participants by ACS COR positivity[25] will provide real-world data on the efficacy of a strategy that screens South African adults with the COR signature and provides preventive therapy to those who are COR-positive (clinicaltrials.gov identifier: NCT02735590). Evaluation of the costs and benefits of such strategies should be carefully evaluated in future implementation studies of RISK4[36]. One benefit might be a strong motivation for both health care personnel and patients alike to initiate and complete preventative treatment in the face of a positive COR test. The potential need for repeat test performances also needs to be evaluated.

We identified several transcript pairs that recapitulated the predictive performance of the RISK4 signature and reflected complementary signals in predicting risk of TB progression. RISK4 comprises GAS6 and SEPT4 (up-regulated), and BLK and CD1C (down-regulated). Interestingly, CD1C and growth arrest-specific 6 (GAS6, activating ligand of AXL) are expressed in two distinct dendritic cell (DC) subsets, where GAS6 expression defines a newly characterized AXL^{+}SIGLEC6^{+} DC population[38], suggesting that TB pathogenesis may involve redistribution of circulating DC subsets. The Septin 4 (SEPT4)

protein has anti-apoptotic functions and its deletion improved wound healing in mice[39], suggesting a possible association with lung healing during TB progression. BLK is a B cell receptor kinase, and its downregulation is consistent with reduced B cell proportions in blood during TB[19, 40]. The most generalizable pair defined in our meta-analysis showed up-regulation of the complement C1q C-chain (C1QC), and down-regulation of T-cell receptor alpha variable gene 27 (TRAV27). Interestingly, complement pathway genes are markedly up-regulated following *M.tb* infection of non-human primates[41], consistent with the up-regulation of C1QC/TRAV27 at baseline in the HHC. Complement activation is also observed early during human progression to TB[40] while C1q is down-regulated early after starting TB treatment[22], suggesting that C1q may be a proxy of early TB pathology. Conversely, down-regulation of TRAV27, and several other T-cell genes **(Supplementary Table 18)**, is likely associated with the overall decrease in peripheral T-cell frequencies and their associated gene expression modules during TB progression, potentially due to migration of T-cells to the disease site[19, 21, 40]. The simple C1QC/TRAV27 signal may thus be a read-out of TB risk following initial exposure to a pulmonary TB case, which is more synchronized in a HHC study design, even though prior exposure to *M.tb* cannot be ruled out in our GC6-74 study, and progression to TB disease within the first three months of the observation period were excluded from the analysis. This may explain why C1QC/TRAV27 signal was less predictive in the natural history cohort of *M.tb*-infected adolescents, where the time of *M.tb* exposure was unspecified. Early clinical studies suggest that recent exposure to *M.tb*, indicated

by TST conversion, can correlate with symptoms consistent with febrile disease, such as fever and erythema nodosum[42, 43], markers of systemic inflammation. C1QC/TRAV27 may reflect this inflammatory response induced by failed containment of *M.tb* following recent exposure.

Overall, our study identifies and validates a simple PCR-based test from accessible blood samples that predicts TB in heterogeneous African populations with intermediate to high TB burdens[14, 15]. Such a test can potentially be developed into a screening test for risk of progression during TB contact investigation, implemented by national public health structures[13, 35, 36]. The next steps include assessment of the performance of RISK4 and the 2-transcript C1QC/TRAV27 signature in other settings, including non-African populations and to determine the feasibility of developing a near-patient test for targeted intervention.

Table 1: Baseline demographic characteristics of progressors enrolled and matched non-progressor controls in the 4 African household contact cohorts. n: number of individuals, IQR: interquartile range.

Site	South Africa	The Gambia	Ethiopia	Uganda
HIV- HHC, n	1,197	1,948	818	499
Progressors, n Incidence, %	43 3.6	34 1.7	12 1.5	11 2.2
Median age, years (IQR)				
Progressors	25 (18-41)	22.5 (20-30.75)	23 (19.75-27)	23 (18-36)
Non-progressors	24 (18-38)	24 (18-30.25)	25 (20-35)	27 (19-38.75)
Male, %				
Progressors	41.9	44.1	33.3	54.5
Non-progressors	40.7	44.1	35.4	54.5
Median time to TB, months (IQR)				
Progressors	7 (5-17)	10.5 (7-18.75)	10 (6.5-15)	7 (5-11)

Figure Legends

Figure 1: Consort diagram describing the inclusion and exclusion of participants from the different African cohorts in the Grand Challenges 6-74 household contact study: Stellenbosch University in South Africa (SUN), Armauer Hansen Research Institute in Ethiopia (AHRI), Makerere University in Uganda (MAK), Medical Research Council in The Gambia (MRC), and the external validation natural history study of South African Adolescents (ACS) in training predictive transcriptomic biomarker for TB progression. "QC Excluded" pertains to samples excluded because they did not meet the minimum quality control requirement for RNA-sequencing of an RNA yield ≥200ng and an RNA Integrity number (RIN) ≥7.

Figure 2: Site-specific Feature Selection and Translation to RT-PCR. (A) Receiver Operating Characteristic (ROC) Curve for Leave-One-Out Cross-Validation (LOOCV) of South Africa (blue; AUC=0.86 [0.79-0.94], $p=8.4x10^{-10}$) vs. The Gambia-trained prospective signature (red; AUC=0.59 [95% CI: 0.46-0.73], *p=0.06*) in South African training set; samples listed in Supplementary Tables 11A and 11B. **(B)** ROC curves for LOOCV of The Gambia (blue; AUC=0.77 [0.66-0.88], $p=2.5x10^{-5}$) vs. South Africa prospective signature (red; AUC=0.66 [0.54-0.77], $p=8.8X10^{-3}$) in The Gambia training set containing 26 progressor and 76 non-progressor samples. **(C and D)** Heatmaps showing the expression of each splice junction in the South Africa **(C)** and The Gambia **(D)** signatures in non-progressors (left columns), progressors 1-2 years before diagnosis (middle

columns), and progressors 0–1 years before diagnosis (right columns). For each group of samples, the central column is the mean fold expression change vs non-progressors, while left/right columns in each group correspond to mean -/+ standard error of the mean. Each row corresponds to a splice junction, and genes with multiple rows are represented by multiple splice junctions in the signature.

Figure 3: Validation of a multi-cohort 4-gene (RISK4) signature derived from the South African and Gambia training sets. (A) Expression ratio of gene pairs in the RISK4 signature, in South Africa (top) and The Gambia (bottom) training set: non-progressors (left columns), progressors 1–2 years before diagnosis (middle columns), and progressors 0–1 (right columns) years before diagnosis. In each group, the central column is the mean fold expression over non-progressors, while left/right columns in each group correspond to mean -/+ standard error of the mean. **(B)** ROC curves for blind predictions of RISK4 on test set samples of all sites (black: AUC=0.67 [0.57-0.77], $p=2.6X10^{-4}$), South Africa (red: AUC=0.72 [0.53-0.92], $p=6.3X10^{-3}$), The Gambia (blue: AUC=0.72 [0.55-0.88], $p=5.4X10^{-3}$), and Ethiopia (green: AUC=0.67 [0.5-0.83], $p=0.02$). **(C)** Performance of RISK4 signature in test set samples taken within one year of diagnosis (red; AUC=0.66 [0.55-0.78], $p=1.9X10^{-3}$; 30 progressor samples, 201 non-progressor samples) or 1-2 years before diagnosis (blue; AUC=0.69 [0.51-0.86], $p=0.015$; 12 progressor samples, 201 non-progressor samples). **(D)** ROC curve of RISK4 on all baseline test set samples (AUC=0.69 [0.52-0.86],

p=$4.8X10^{-3}$). **(E)** ROC curve blind prediction of RISK4 in latently *M.tb*-infected South African adolescents (AUC=0.69 [0.62-0.76], *p*=$3.4X10^{-7}$).

Figure 4: Comparison of PCR-adapted signatures: RISK4, COR and TB diagnostic signatures. (A) ROC curves for blind predictions of RISK4 (Black: AUC=0.67 [0.57-0.77], *p*=$2.6X10^{-4}$), DIAG3 (red: AUC=0.68 [0.59-0.78], *p*=$8.4X10^{-5}$), DIAG4 (blue: AUC=0.64 [0.53-0.74], *p*=$2.6X10^{-3}$) and ACS COR (green: AUC=0.66 [0.55-0.76], *p*=$5.8X10^{-4}$) in all test set samples. **(B-D)** Blind prediction of PCR-adapted signatures: DIAG3 (B: South Africa AUC=0.66 [0.47-0.84], The Gambia AUC=0.6 [0.45-0.77] and Ethiopia AUC=0.78 [0.64-0.92]), DIAG4 (C: South Africa AUC=0.77 [0.62-0.91], The Gambia AUC=0.52 [0.33-0.71] and Ethiopia AUC=0.64 [0.46-0.83]) and RISK16 (D: South Africa AUC=0.82 [0.71-0.92], The Gambia AUC=0.56 [0.37-0.75] and Ethiopia AUC=0.6 [0.41-0.79]). South Africa, The Gambia and Ethiopia AUCs are depicted in red, blue and green, respectively.

Figure 5: Gene pairs to predict TB progression in African cohorts. Ratios of C1QC/TRAV27 and ANKRD22/OBSPL10 plotted on samples from South Africa **(A)**, The Gambia **(B)**, and Ethiopia **(C)** along with an optimal discriminant (dashed line; optimizes sum of sensitivity and specificity) separating progressors (orange) from non-progressors (blue). On each cohort, the two pairs provide complementary information; p-values correspond to Chi-square complementation analysis in Supplementary Table 16. **(D)** ROC curves showing the ability of the

GC6-trained C1QC/TRAV27 (solid; AUC=0.57 [0.49-0.64], *p=0.042*), ANKRD22/OBSPL10 (dashed; AUC=0.75 [0.68-0.81], $p=2.86x10^{-11}$), and a linear combination of C1QC/TRAV27 and ANKRD22/OBSPL10 (dotted; AUC=0.69 [0.61-0.76], $p=4.3X10^{-07}$) models to predict TB disease progression on in the ACS cohort. **(F and G)** Log-ratios of expression (mean +/- 95% confidence interval) for ANKRD22/OBSPL10 **(F)** and C1QC/TRAV27 **(G)** are plotted as a function of time to diagnosis, for both GC6 (blue) and ACS (red) progressor samples. Comparison of C1QC/TRAV27 expression at 19-24 months before diagnosis, between the GC6-74 HHC and ACS cohorts was statistically significantly different ($p=3X10^{-3}$) using the Mann-Whitney *U* test.

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Fig 1

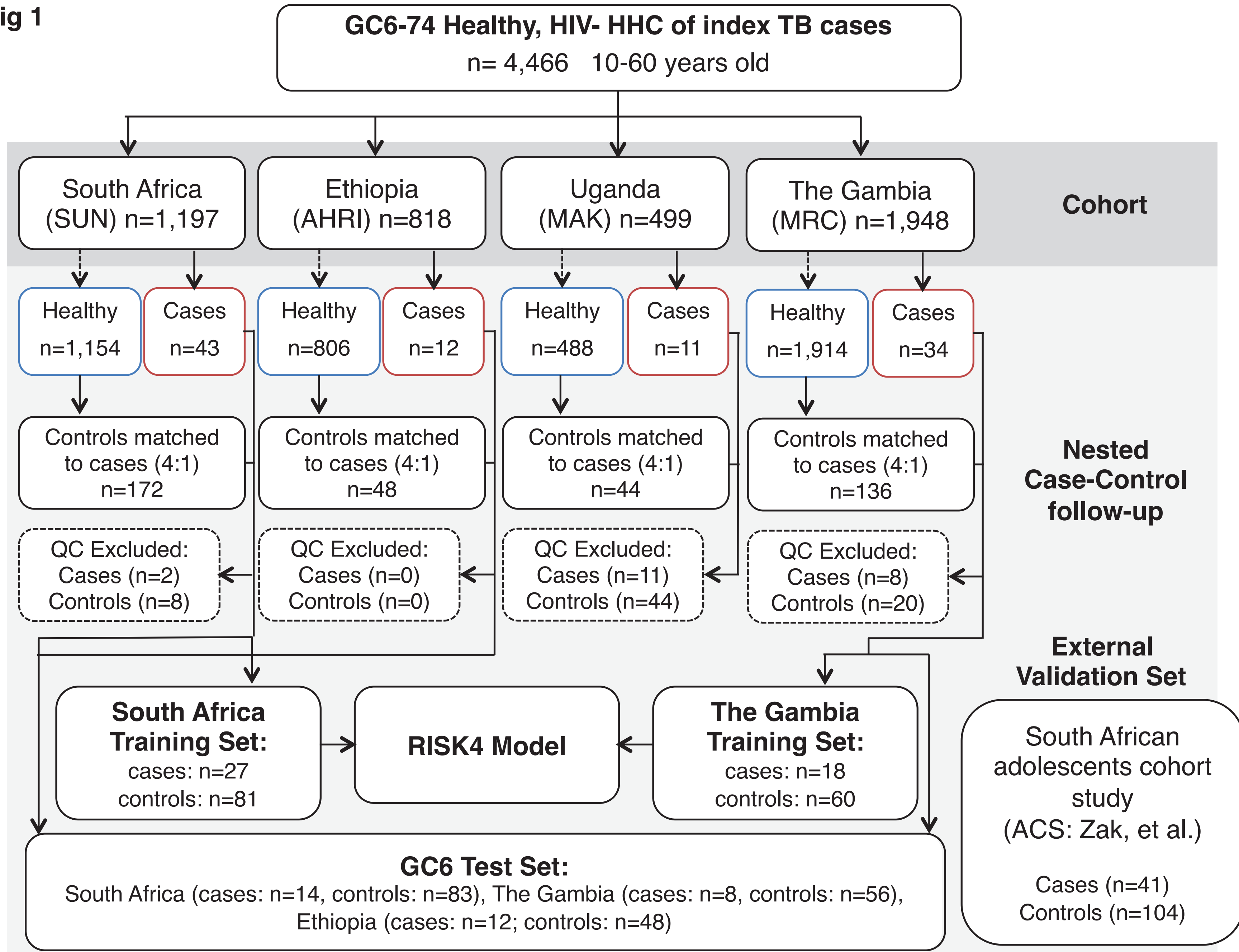
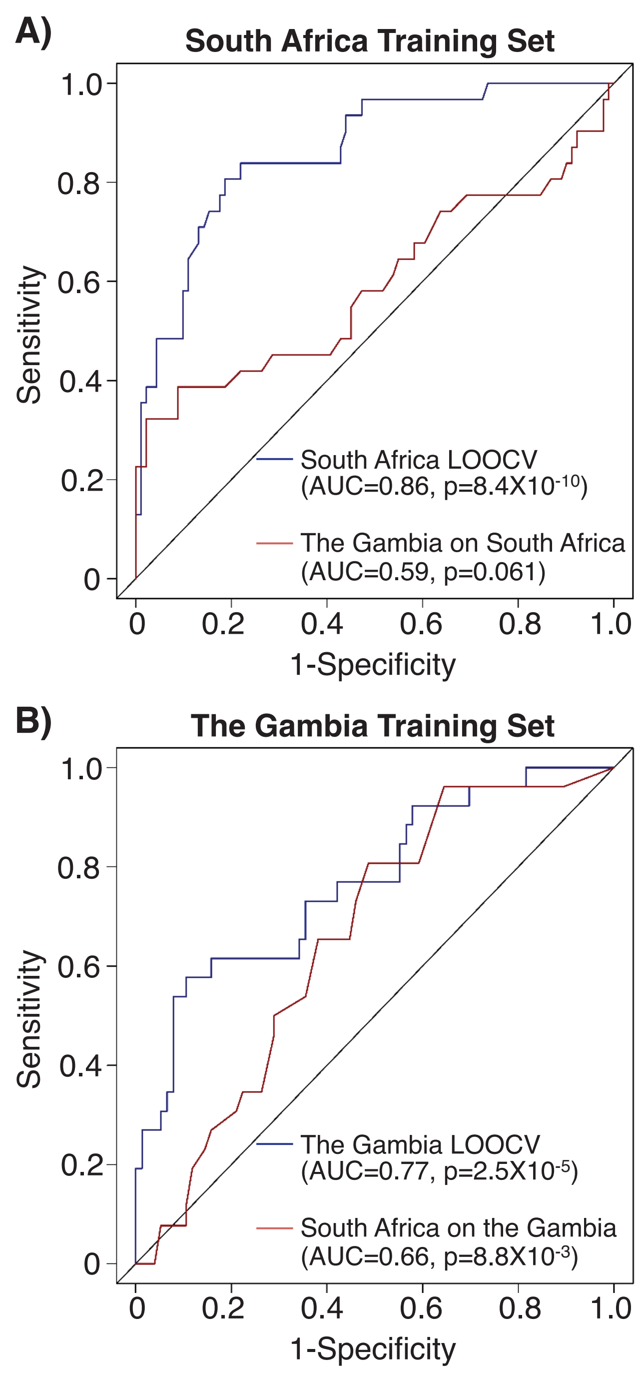


Fig 2



C)

C14orf159
C1orf131
CD63
CRTAM
DCAF17
DDX47
DGCR8
DOCK9
IGFLR1
KIAA0586
KLRK1
KNTC1
LINC00969
LZTFL1
MFSD7
MMP8
MSRB2
NR1H2
OMA1
OXNAD1
PLAUR
POLA1
PSMB10
QRSL1
RBCK1
RENBP
SIPA1L1
SLC2A5
THUMPD2
ZNF14
ZNF286A

1
0.5
0
-0.5
-1

Non-progressors
1 – 2 years
0 – 1 years

South Africa

D)

ACBD4
ADAMTSL4
ANKRD22
BLK
CD1C
DRAM2
DUSP3
ENO2
FAM20A
FCGR1B
FFAR3
FLVCR2
GAS6
HAUS5
NA
NRG1
PHF1
RASGRP2
SEPT4
SLC22A17
SLC26A8
SORT1
SPHK1
VCAN

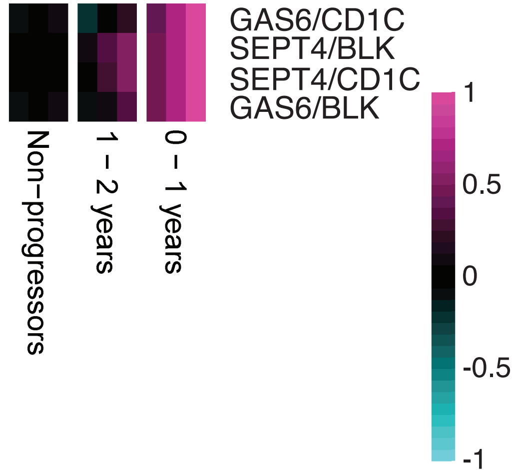
1
0.5
0
-0.5
-1

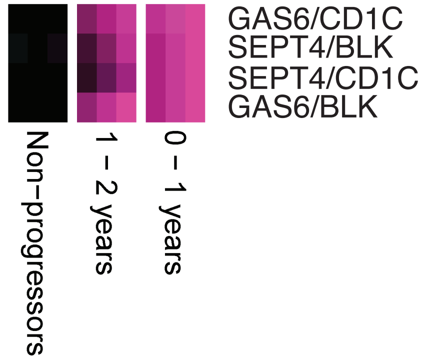
Non-progressors
1 – 2 years
0 – 1 years

The Gambia

Fig 3

A) RISK4 Signature





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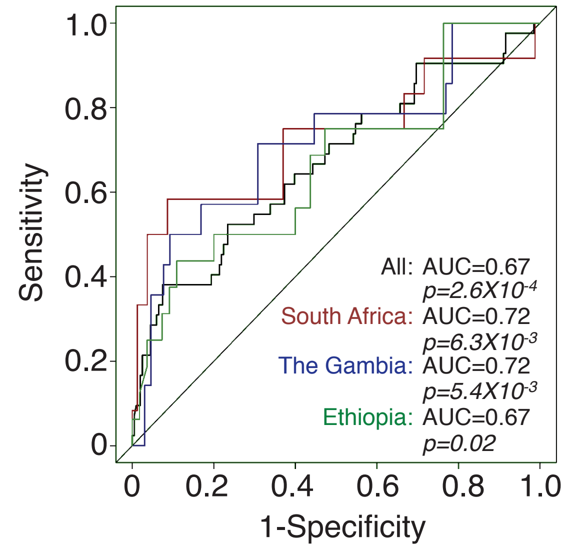

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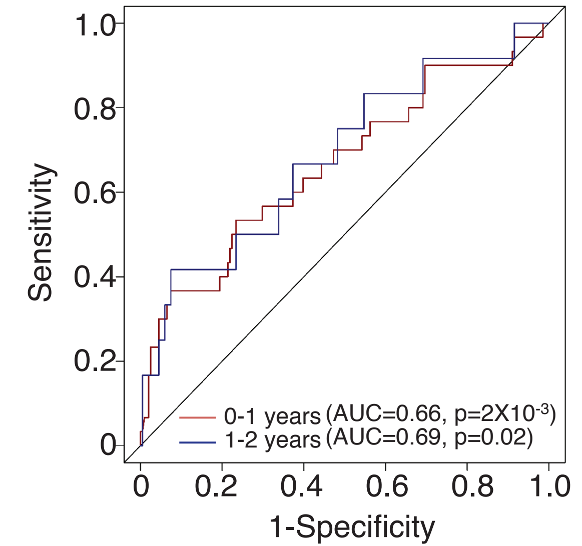

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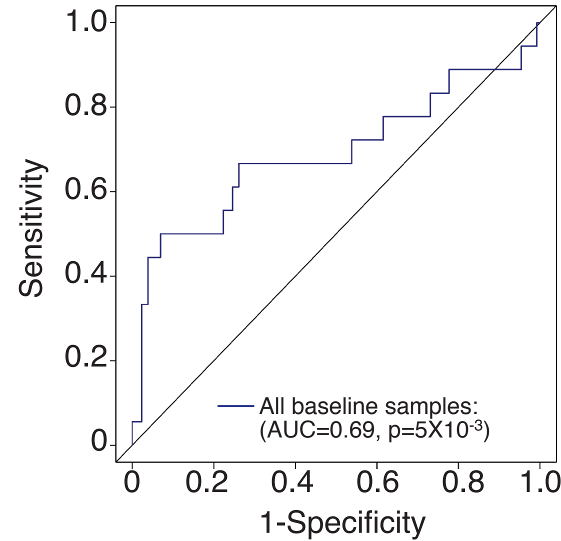

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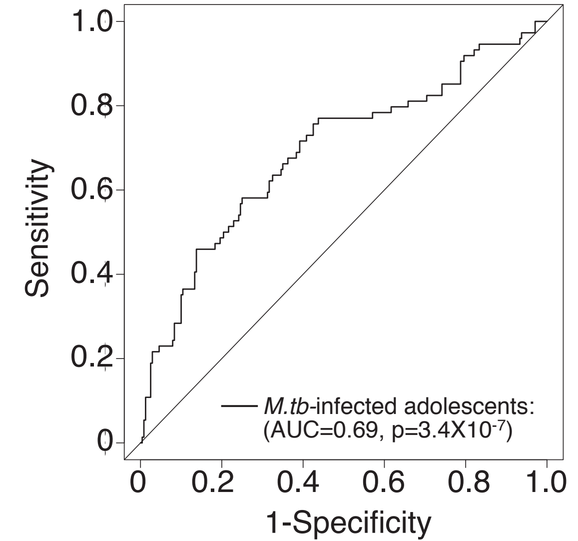
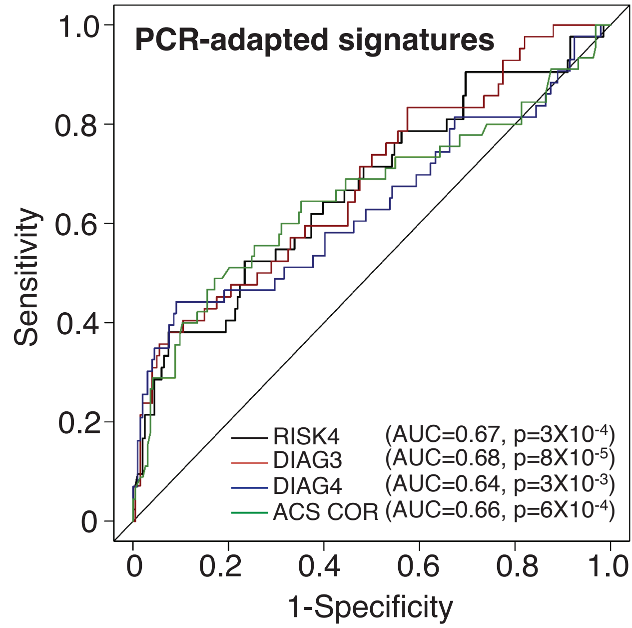
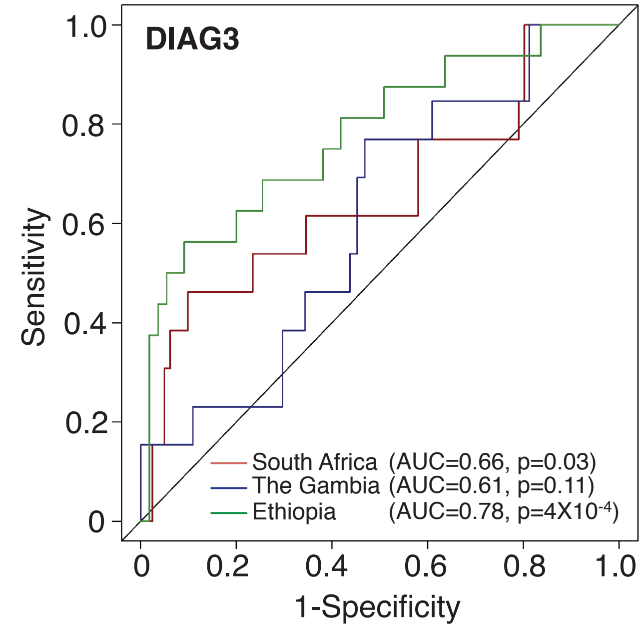
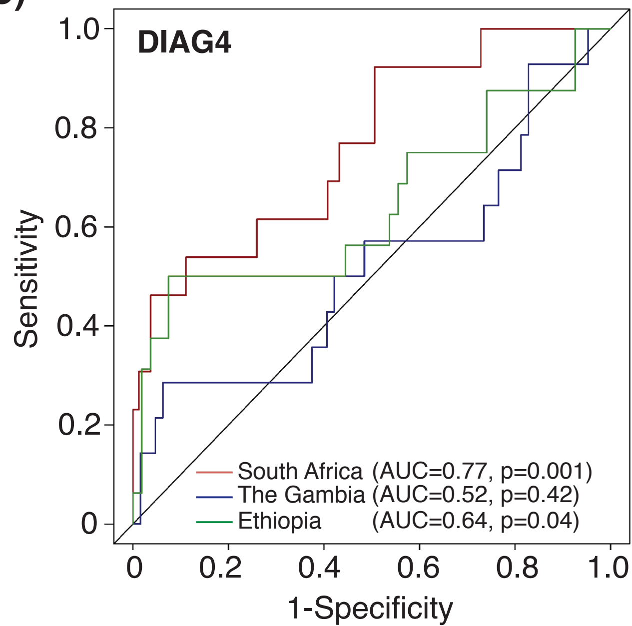


Fig 4







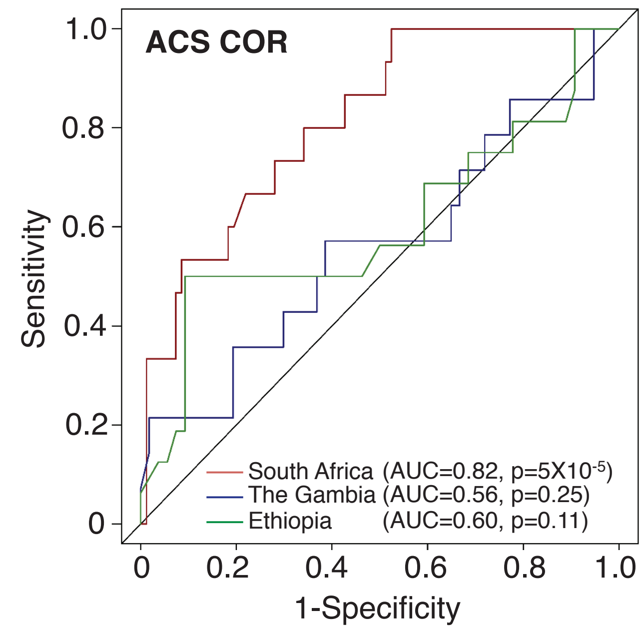


Fig 5