A blood RNA signature for tuberculosis disease risk: a prospective cohort study

Daniel E Zak, Adam Penn-Nicholson*, Thomas J Scriba*, Ethan Thompson†, Sara Suliman†, Lynn M Amon, Hassan Mahomed, Mzwandile Erasmus, Wendy Whatney, Gregory D Hussey, Deborah Abrahams, Fazlin Kafaar, Tony Hawkridge, Suzanne Verver, E Jane Hughes, Martin Ota, Jayne Sutherland, Rawleigh Howe, Hazel M Dockrell, W Henry Boom, Bonnie Thiel, Tom H M Ottenhoff, Harriet Mayanja-Kizza, Amelia C Crampin, Katrina Downing, Mark Hatherill, Joe Valvo, Smitha Shankar, Shreemanta K Parida, Stefan H E Kaufmann, Gerhard Walzl, Alan Aderem, Willem A Hanekom, for the ACS and GC6-74 cohort study groups‡*

Lancet 2016; 387: 2312–22

Published Online March 23, 2016 http://dx.doi.org/10.1016/S0140-6736(15)01316-1

See Comment page 2268

*Contributed equally

†Contributed equally

‡Members listed at the end of paper

The Center for Infectious Disease Research, Seattle, WA, USA (D E Zak PhD, E Thompson PhD, L M Amon PhD, J Valvo BS, S Shankar MS, A Aderem PhD); **South African Tuberculosis Vaccine Initiative, Institute of Infectious Disease and Molecular Medicine & Department of Paediatrics and Child Health, University of Cape Town, Cape Town, South Africa** (A Penn-Nicholson PhD, T J Scriba PhD, S Suliman PhD, H Mahomed MD, M Erasmus BSc, W Whatney BScHons, Prof G D Hussey FFCH(SA), D Abrahams DipMT, F Kafaar DipNur, T Hawkridge FCPHM, E J Hughes BScHons, K Downing PhD, M Hatherill MD, Prof W A Hanekom FCP[SA]); **KNCV Tuberculosis Foundation, The Hague, Netherlands** (S Verver PhD); **Vaccines & Immunity, Medical Research Council Unit, Fajara, The Gambia** (M Ota MD, J Sutherland PhD); **Immunology Unit, Armauer Hansen Research Institute, Addis Ababa, Ethiopia** (R Howe, MD); **Department of Immunology and Infection, London School of Hygiene & Tropical Medicine, London, UK** (Prof H M Dockrell PhD, A C Crampin FFPHM); **Tuberculosis Research Unit, Case Western Reserve University, Cleveland, OH, USA**

Summary

Background Identification of blood biomarkers that prospectively predict progression of *Mycobacterium tuberculosis* infection to tuberculosis disease might lead to interventions that combat the tuberculosis epidemic. We aimed to assess whether global gene expression measured in whole blood of healthy people allowed identification of prospective signatures of risk of active tuberculosis disease.

Methods In this prospective cohort study, we followed up healthy, South African adolescents aged 12–18 years from the adolescent cohort study (ACS) who were infected with *M tuberculosis* for 2 years. We collected blood samples from study participants every 6 months and monitored the adolescents for progression to tuberculosis disease. A prospective signature of risk was derived from whole blood RNA sequencing data by comparing participants who developed active tuberculosis disease (progressors) with those who remained healthy (matched controls). After adaptation to multiplex quantitative real-time PCR (qRT-PCR), the signature was used to predict tuberculosis disease in untouched adolescent samples and in samples from independent cohorts of South African and Gambian adult progressors and controls. Participants of the independent cohorts were household contacts of adults with active pulmonary tuberculosis disease.

Findings Between July 6, 2005, and April 23, 2007, we enrolled 6363 participants from the ACS study and 4466 from independent South African and Gambian cohorts. 46 progressors and 107 matched controls were identified in the ACS cohort. A 16 gene signature of risk was identified. The signature predicted tuberculosis progression with a sensitivity of 66·1% (95% CI 63·2–68·9) and a specificity of 80·6% (79·2–82·0) in the 12 months preceding tuberculosis diagnosis. The risk signature was validated in an untouched group of adolescents (p=0·018 for RNA sequencing and p=0·0095 for qRT-PCR) and in the independent South African and Gambian cohorts (p values <0·0001 by qRT-PCR) with a sensitivity of 53·7% (42·6–64·3) and a specificity of 82·8% (76·7–86) in the 12 months preceding tuberculosis.

Interpretation The whole blood tuberculosis risk signature prospectively identified people at risk of developing active tuberculosis, opening the possibility for targeted intervention to prevent the disease.

Funding Bill & Melinda Gates Foundation, the National Institutes of Health, Aeras, the European Union, and the South African Medical Research Council.

Introduction

A third of the population worldwide is infected with *Mycobacterium tuberculosis*,[1] but less than 10% of these individuals will progress to have active tuberculosis disease during their lifetime; most individuals will remain healthy.[2–6] Risk of progression is associated with age,[7] comorbidities such as HIV infection and diabetes mellitus, socioeconomic and nutritional compromise, and therapy with immune modulatory drugs such as tumour necrosis factor inhibitors, among others.[8,9] Current assays for determining the presence of *M tuberculosis* infection, such as an interferon gamma release assay (IGRA) or tuberculin skin test (TST), cannot predict which infected individuals will develop active tuberculosis.

Previous systems biology approaches have identified diagnostic signatures that discriminate tuberculosis disease from latent *M tuberculosis* infection and from other disease states.[10–21] For example, Berry and colleagues[12] identified and validated a 393 gene signature that allowed differentiation of people with active tuberculosis disease and latent infection. Anderson and colleagues[14] identified and validated a 53 gene signature that distinguished active tuberculosis from other diseases in African children with or without HIV infection. By contrast with the published diagnostic studies, our focus was on prospective signatures of risk that could be identified in healthy individuals up to 2 years before clinical tuberculosis disease manifests.

Knowledge gained from this signature could lead to targeted antimicrobial therapy to prevent tuberculosis disease, as treating all people who are latently infected in endemic countries for 6–9 months is not feasible.

Research in context

Evidence before this study

We searched the PubMed database for studies published before July 1, 2015, using the search criteria "tuberculosis AND risk AND blood AND (RNA OR microarray OR transcriptome OR RNA-Seq)". The resulting scientific literature included several substantial analyses comparing the blood RNA profiles of individuals with active tuberculosis disease and healthy individuals. These important studies have established that the tuberculosis disease state is reflected in the blood RNA profile of the patient with tuberculosis with ongoing disease. The resulting literature also included several small studies reporting candidate host markers for tuberculosis disease risk that have not been rigorously assessed in independent cohorts. Repeating the search without the "(RNA OR microarray OR transcriptome OR RNA-Seq)" term yielded literature with established risk factors for tuberculosis disease, which have been summarised in several reviews. Despite these important studies and known tuberculosis risk factors, it is not possible to predict which individuals infected with *Mycobacterium tuberculosis* will develop active tuberculosis with tools.

Added value of this study

Our study expands the previous findings by being the first large-scale search for prospective correlates of risk of tuberculosis in healthy individuals before the onset of disease. We used unbiased high-throughput screening of host blood RNA profiles to identify new signatures of risk for tuberculosis. These signatures were confirmed in the original cohort with targeted assays; these targeted assays were then successfully used to predict tuberculosis disease progression in two independent cohorts. Further meta-analyses of published datasets showed that the prognostic signatures might simultaneously serve as diagnostic signatures for tuberculosis.

Implications of all the evidence

Our study provides the first demonstration that host blood RNA signatures can be used to predict progression to active tuberculosis disease in healthy individuals that are latently infected with or exposed to the *M tuberculosis* pathogen. These findings will result in further follow-up studies to assess whether the prognostic signatures can be used to prevent tuberculosis disease through targeted prophylactic treatment. Additional follow-up studies might focus on optimising the practical measurement of the signatures and understanding the biological significance of the host genes implicated by the signatures.

(Prof W H Boom MD, B Thiel MS); **Department of Infectious Diseases, Leiden University Medical Center, Leiden, Netherlands** (Prof T H M Ottenhoff MD); **Department of Medicine and Department of Microbiology, Makerere University, Kampala, Uganda** (Prof H Mayanja-Kizza MD); **Karonga Prevention Study, Chilumba, Malawi** (A C Crampin FFPHM); **Department of Immunology, Max Planck Institute for Infection Biology, Berlin, Germany** (S K Parida MD, Prof S H E Kaufmann PhD); **and DST/NRF Centre of Excellence for Biomedical TB Research and MRC Centre for TB Research, Division of Molecular Biology and Human Genetics, Stellenbosch University, Tygerberg, South Africa** (Prof G Walzl MD)

Correspondence to:
Prof Willem A Hanekom, Bill & Melinda Gates Foundation, Seattle, WA 98102, USA
willem.hanekom@gatesfoundation.org

Other potential applications of biomarkers of risk of tuberculosis disease include assessment of response to drug therapy and targeted enrolment into efficacy trials of new tuberculosis vaccines and drugs. In view of the fact that a third of the world's population is latently infected with *M tuberculosis*, our approach constitutes an opportunity to lessen the burden of disease. To this end, we aimed to assess whether global gene expression measured in whole blood of healthy people allowed identification of prospective signatures of risk of active tuberculosis disease.

Methods

Study design and participants

We included participants from several cohorts in this analysis. First, we assessed participants aged 12–18 years from the South African adolescent cohort study (ACS) who were infected with *M tuberculosis* to identify and validate a tuberculosis risk signature (figure 1). All adolescents whose parents or legal guardians provided written, informed consent and who provided written, informed assent themselves were enrolled. About half the participants from the ACS cohort were assessed at enrolment and every 6 months during 2 year follow-up; the other half were assessed at baseline and at 2 years. At enrolment and at each visit, clinical data were collected and 2·5 mL blood was collected directly into PAXgene blood RNA tubes (PreAnalytiX, Hombrechtikon, Switzerland), which were stored at –20°C.

Only adolescents with latent *M tuberculosis* infection at enrolment were included in the analysis aimed at identification of a tuberculosis risk signature. Latent *M tuberculosis* infection was diagnosed by a positive QuantiFERON TB gold in-tube assay (Cellestis, Chadstone, Australia; >0·35 IU/mL) or a positive tuberculin skin test (0·1 mL dose of purified protein derivative RT-23, 2-TU, Staten Serum Institute, Denmark; >10 mm), or both. According to South African policy, adolescents positive on these tests were not given therapy to prevent tuberculosis disease.[22] Adolescents who developed active tuberculosis disease during follow-up were included as progressors. Tuberculosis was defined as intrathoracic disease, with either two sputum smears positive for acid-fast bacilli or one positive sputum culture confirmed as *M tuberculosis* complex (mycobacterial growth indicator tube, BD BioSciences, NJ, USA). For each progressor, two matched controls that remained healthy during follow-up were selected and matched by age at enrolment, sex, ethnic origin, school of attendance, and presence or absence of previous episodes of tuberculosis disease. Participants were excluded if they developed tuberculosis disease within 6 months of enrolment to exclude early asymptomatic disease that could have been present at the time of assessment, or if they were HIV-positive. Before analysis, the ACS progressors and controls were randomly divided into training and test sets, at a ratio of 3:1 using the randomisation function in Excel.

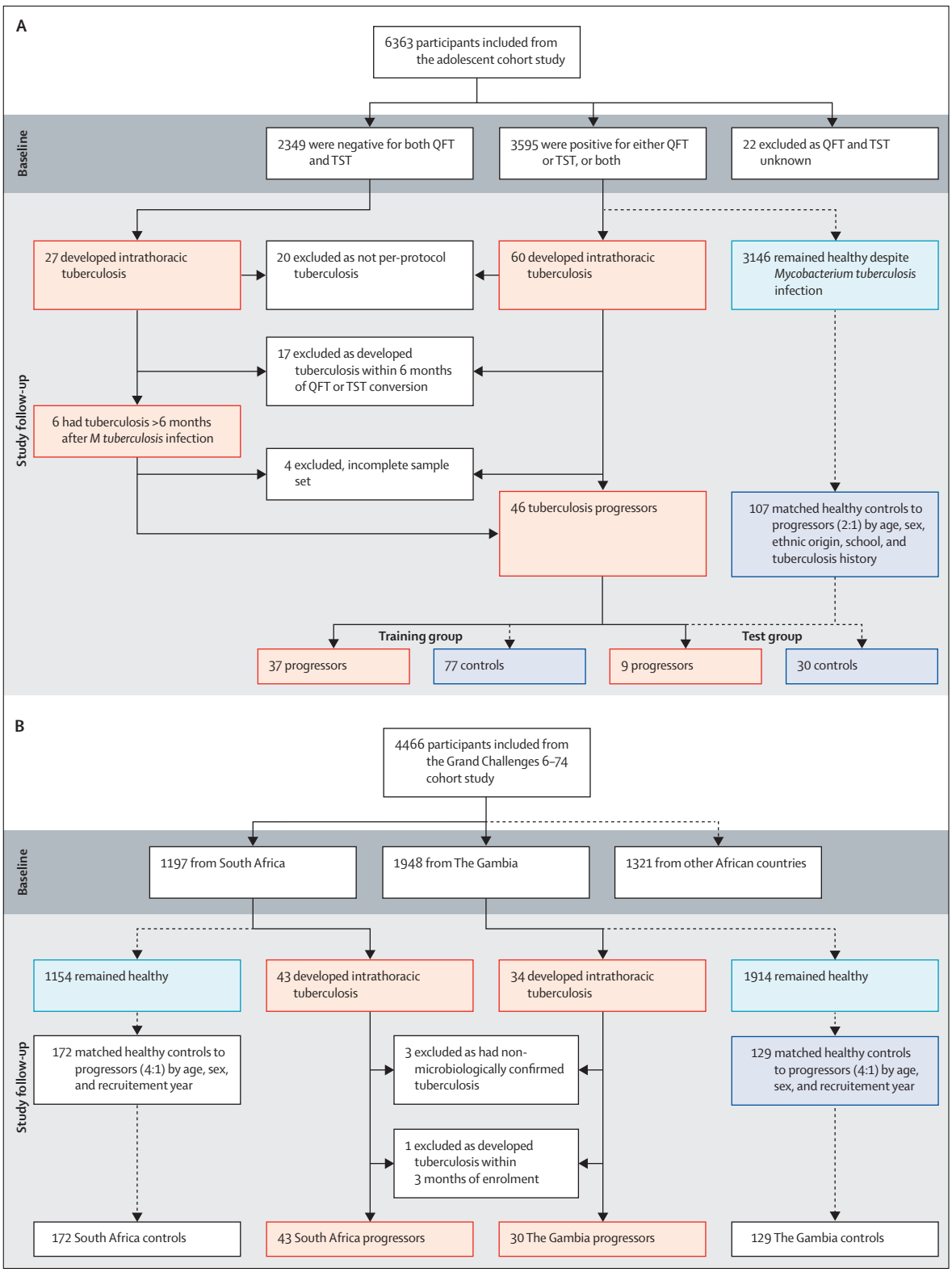


Figure 1: **The adolescent cohort study and the Grand Challenges 6-74 study cohorts for the discovery and validation of the tuberculosis risk signature**
(A) Inclusion and exclusion of participants from the adolescent cohort study and assignment of eligible progressors and controls to the training and test sets. (B) Inclusion and exclusion of adult household contacts of patients with lung tuberculosis from the Grand Challenges 6-74 study cohorts, and assignment of eligible progressors and controls. QFT=QuantiFERON TB gold in-tube assay. TST=tuberculin skin test.

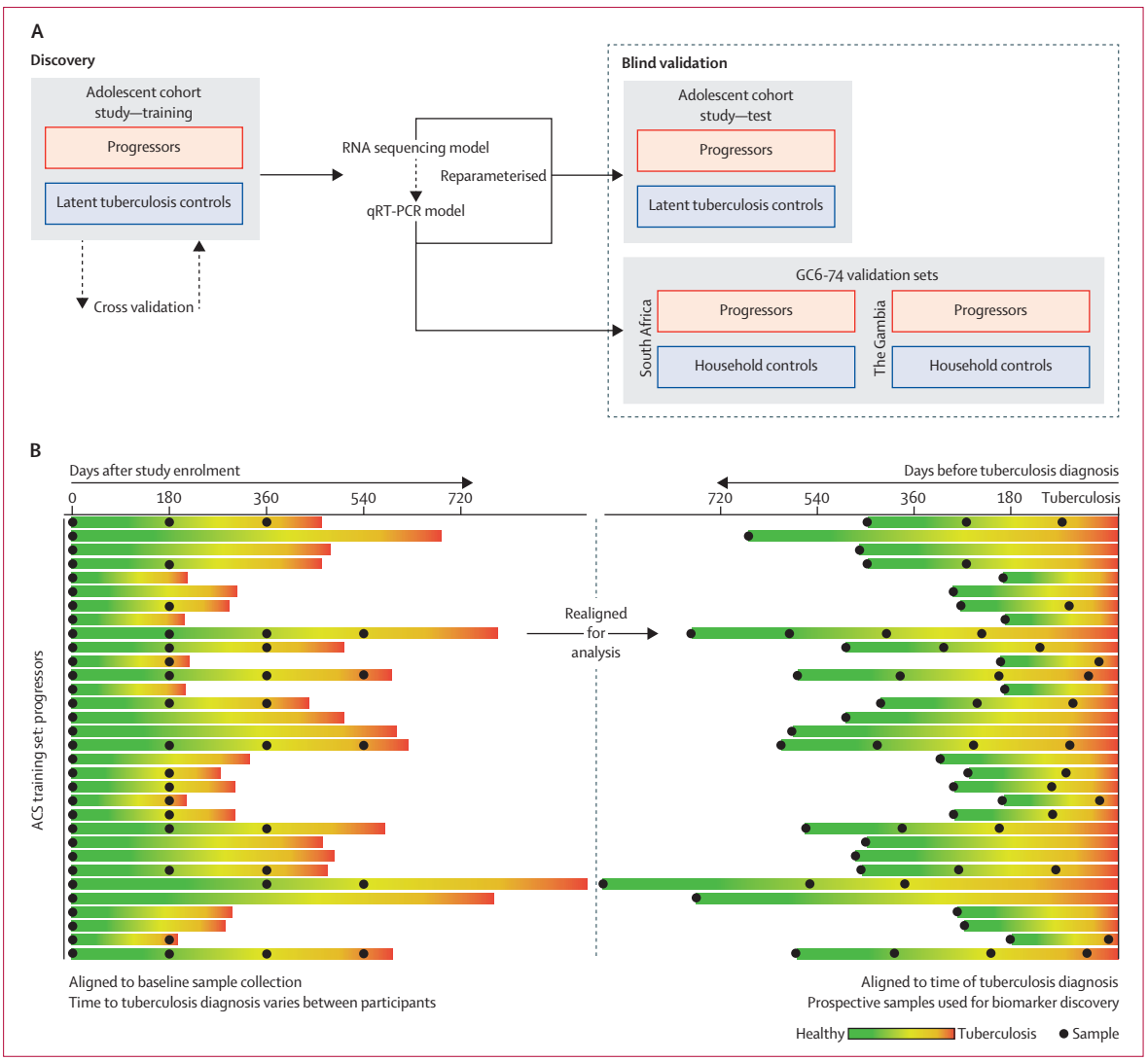


Figure 2: **Strategy for discovery and validation of the tuberculosis risk signature**
(A) Flow diagram for the discovery and validation of the tuberculosis risk signature. The tuberculosis risk signature was obtained by data mining of a whole blood RNA sequencing dataset generated from the adolescent cohort study training set. The predictive potential of the risk signature was assessed by rigorous cross validation. The tuberculosis risk signature was adapted to qRT-PCR, and then the RNA sequencing and qRT-PCR versions of the signature were used to predict tuberculosis progression in untouched blinded samples from the adolescent cohort study test set. The qRT-PCR-based tuberculosis risk signature was then used to predict tuberculosis progression using untouched blinded samples from the South African and Gambian cohorts of GC6-74. (B) Synchronisation of the adolescent cohort study training set in terms of the clinical outcome. To ensure optimal extraction of a tuberculosis risk signature from the adolescent cohort study training set, the timescale of the RNA sequencing dataset was realigned according to tuberculosis diagnosis instead of study enrolment, allowing gene expression differences to be measured before disease diagnosis. Each progressor within the adolescent cohort study training set is represented by a horizontal bar. The length of the bar represents the number of days between study enrolment and diagnosis with active tuberculosis. During follow-up, each progressor transitioned from an asymptomatic healthy state (green) to pulmonary disease (red). The left graph shows alignment of PAXgene sample collection (black points) with respect to study enrolment. The right graph shows alignment of PAXgene sample collection with respect to diagnosis with active tuberculosis, for use in analysis. qRT-PCR=quantitative real-time PCR. GC6-74=Grand Challenges 6-74 study. ACS=adolescent cohort study.

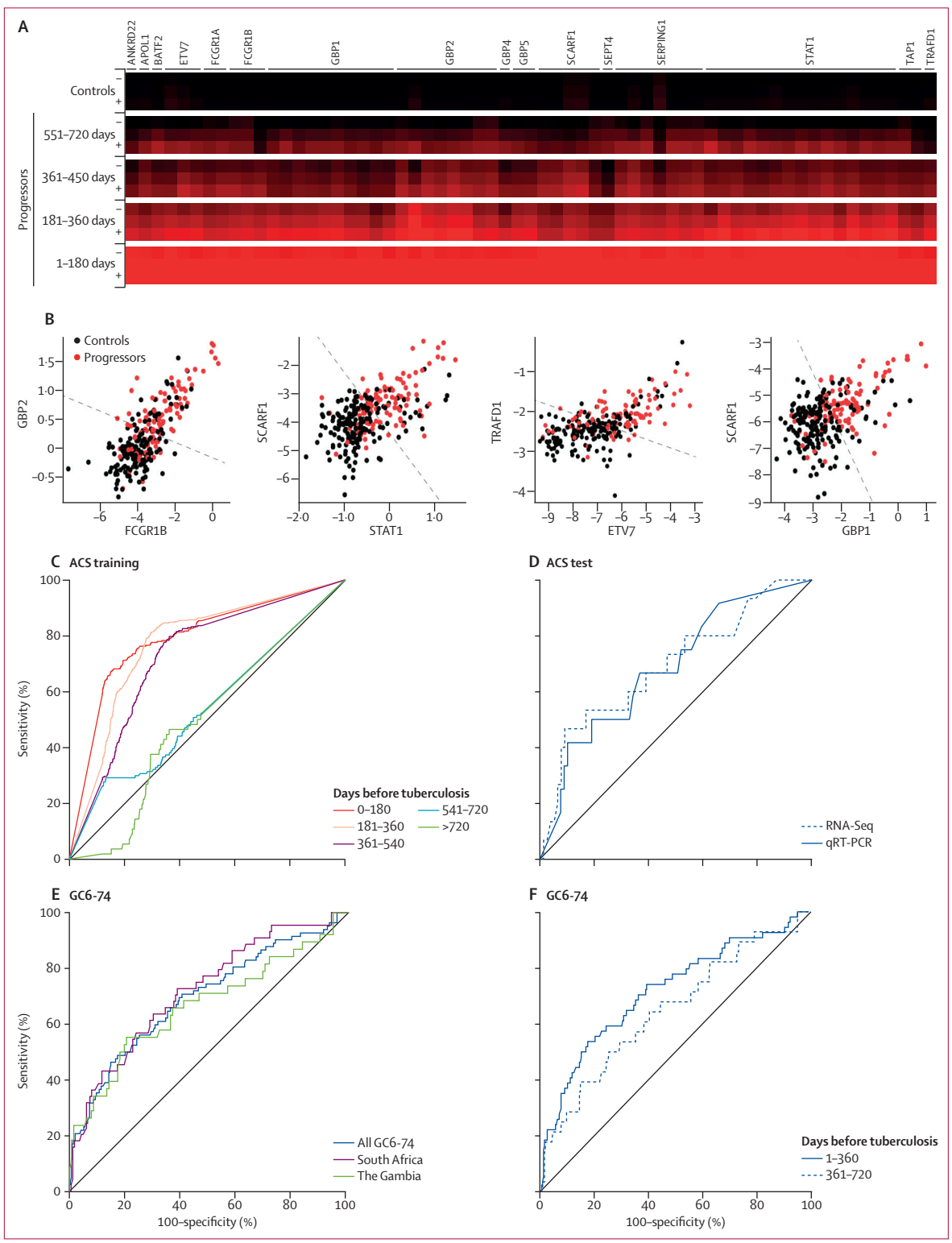
The other cohorts consisted of South African and Gambian participants from the Grand Challenges 6-74 study (GC6-74) who were enrolled to independently validate the tuberculosis risk signature (figure 1). Briefly, from a parent GC6-74 cohort, HIV-negative people aged 10–60 years who had household exposure to an adult with sputum smear positive tuberculosis disease were enrolled to this study. At baseline (both sites), at 6 months (The Gambia only), and at 18 months (both sites), participants were assessed clinically and blood was collected and stored in PAXgene tubes. Follow-up continued for 2 years, and concluded on Nov 18, 2012. Among GC6-74 participants, progressors had intrathoracic tuberculosis, defined on the basis of sputum culture, smear microscopy, and clinical signs. For each progressor, four controls were matched

For more on the **Grand Challenges study** see http://www.case.edu/affil/tbru/collaborations_gates.html

See Online for appendices

according to recruitment region, age category (≤18 years, 19–25 years, 26–35 years, or ≥36 years), sex, and year of enrolment.

The study protocols were approved by the relevant human research ethics committees (appendix 1). Written informed consent was obtained from participants. For



adolescents, consent was obtained from parents or legal guardians of adolescents and written informed assent from each adolescent. In both studies, participants with diagnosed or suspected tuberculosis disease were referred to a study-independent public health physician for treatment according to national tuberculosis control programmes of the country involved.

Procedures

The analytical approach to identify and validate the tuberculosis risk signature is shown (figure 2). The tuberculosis risk signature was derived from mining RNA sequencing data generated from the ACS training set. The RNA sequencing based tuberculosis risk signature was then adapted to the quantitative real-time PCR (qRT-PCR) platform. The RNA sequencing and qRT-PCR-based signature of risk was validated by blind prediction on untouched samples from the ACS test set. The qRT-PCR-based tuberculosis risk signature was also validated by blind prediction on independent GC6-74 cohort samples from South Africa and The Gambia.

For RNA sequencing analysis of the ACS training and test sets, RNA was extracted from PAXgene tubes of the ACS training set. Globin transcript depletion (GlobinClear, ThermoFisher Scientific, MA, USA) was followed by cDNA library preparation using the Illumina mRNA-Seq sample preparation kit (Illumina, CA, USA). RNA sequencing was done by Expression Analysis, Q2 Solutions, NC, USA, at 30 million 50 bp paired-end reads, on Illumina HiSeq-2000 sequencers. Read pairs were aligned to the hg19 human genome using gsnap,[23] which generated a table of gene expression abundances for each sample. This gene expression abundance was measured at the level of splice junction counts, which quantifies the frequency of specific mRNA splicing events in expressed genes; this approach would help with translation to qRT-PCR. For simplicity, splice junction expression levels are referred to as "gene expression levels" throughout.

The tuberculosis risk signature was generated and was adapted from the original RNA sequencing-based platform to qRT-PCR by directly matching splice junctions in the signature to commercial TaqMan primer sets (Thermo Fisher Scientific; appendix 1). A complete set of qRT-PCR data for selected primers was generated for ACS training set samples with the BioMark HD multiplex microfluidic instrument (Fluidigm, CA, USA). Variables in the qRT-PCR-based version of the tuberculosis signature were then assigned by fitting the model to the dataset.

RNA sequencing and qRT-PCR analysis of samples from the ACS test set were done as described. Before analysis, all test set samples were assigned random numerical codes that masked study timepoints and progressor and control status. Prediction of tuberculosis risk on the masked ACS test set samples was then done in a fully blinded manner, in parallel with RNA sequencing and qRT-PCR-based versions of the signature.

We blindly predicted the independent GC6-74 validation cohorts using the qRT-PCR-based signature of risk. qRT-PCR analysis of samples from the South African and Gambian cohorts of GC6-74 was done as described less than 1 year after ACS validation analysis. Before analysis, all samples were assigned random numerical codes. Fully blinded predictions were then made with the qRT-PCR-based signature of risk.

To allow assessment of the risk signature for diagnosis of active disease, results from published microarray-based studies of active tuberculosis versus latent disease or other disease were used.[10–14] The signature was adapted from RNA sequencing to the Illumina platform and parameterised using tuberculosis cases and controls latently infected with *M tuberculosis* from the UK training cohort of Berry and colleagues[12] (appendix 1). The locked-down Illumina microarray-based risk signature was used to make predictions in the independent test and validation cohorts from the study by Berry and colleagues,[12] and from the subsequent studies.[10,11,13,14]

Statistical analysis

Statistical and machine learning approaches were applied to discover the signatures of tuberculosis risk. To generate the tuberculosis risk signature, we used an extension of the k-top-scoring pairs (k-TSP) method, which has been used successfully to identify cancer biomarkers.[24,25] The k-TSP approach identifies pairs of genes that discriminate better than either gene would individually.[25] We replaced the k-TSP rank-based gene pair models with the so-called support vector machine (SVM)-based gene pair models for greater flexibility in predictions. This modification is similar to the k-TSP modification proposed by Shi and colleagues,[26] but holds the advantage of retaining the fault-tolerance and parsimony of k-TSP. For analysis,

***Figure 3*: The tuberculosis risk signature and validation by prediction of tuberculosis disease progression in the untouched ACS test set and the independent GC6-74 cohorts**

(A) Heatmap depicting relative expression level of genes comprising the tuberculosis risk signature in progressors compared with controls. Higher expression in progressors relative to controls is indicated by intensity of red colour. Expression is measured in mean (SD). Individual heatmap rows represent distinct splice junctions of individual genes that comprise the signature. Relative expression in each of four 180 day time windows before tuberculosis diagnosis is shown. (B) The tuberculosis risk signature was generated by assessing multiple gene-pair interactions; four representative gene-pair signatures are shown. In each scatterplot, the normalised expression of one gene within the pair is plotted against that of the other gene, for all ACS training set datapoints. The black dots represent control samples, whereas the red dots represent progressor samples. The dotted black line indicates the optimum linear decision boundary for discriminating progressors from controls. (C) Receiver operating characteristic curves depicting the predictive potential of the tuberculosis risk signature for discriminating progressors from controls. Each receiver operating characteristic curve corresponds to a 180 day interval before tuberculosis diagnosis. Prediction performance was assessed by 100 four-to-one training-to-test splits of the ACS training set. (D) Receiver operating characteristic curves for blind prediction of tuberculosis disease progression in untouched ACS test set samples using the RNA sequencing based (dotted line) or qRT-PCR-based (solid line) signature. (E) Blind prediction on the combined GC6-74 cohort (blue), South African cohort (purple) or Gambian cohort (green). (F) Stratification of prediction on the overall GC6-74 cohort by time before tuberculosis diagnosis. ACS=adolescent cohort study. GC6-74=Grand Challenges 6-74 study.

	ROC AUC (95% CI)	Sensitivity (95% CI)	Threshold
By 6 month period			
1–180	0·79 (0·76–0·82)	71·2% (66·6–75·2)	61%
181–360	0·771 (0·75–0·79)	62·9% (59·0–66·4)	61%
361–540	0·726 (0·70–0·76)	47·7% (42·9–52·5)	61%
541–720	0·540 (0·49–0·59)	29·1% (23·1–35·9)	61%
>720	0·496 (0·43–0·56)	5·4% (2·4–13·0)	61%
By 12 month period			
1–360	0·779 (0·76–0·80)	66·1% (63·2–68·9)	61%
360–720	0·647 (0·62–0·673)	37·5% (33·9–41·2)	61%
Total time period	0·743 (0·73–0·76)	58·4% (56·1–60·7)	61%

Sensitivity values are reported at a specificity of 80·0% (95% CI 78·6–81·4). ROC AUC=area under receiver operating characteristic curve. ACS=adolescent cohort study.

Table 1: **Cross-validation performance of the tuberculosis risk signature in the ACS training set by days before tuberculosis diagnosis**

prospective RNA sequencing data of progressors was realigned to the timepoint at which active tuberculosis was diagnosed (figure 2), thereby synchronising the cohort with respect to outcome.

The genes that comprise the final tuberculosis risk signature were selected in two stages, with data from the ACS training set. First, a large set of genes was identified by comparing gene expression in progressors at the most proximal timepoint to diagnosis with that in matched controls. SVM models were trained on these datapoints for all possible pairwise combinations of risk-associated genes. Second, the models were filtered for predictive accuracy with the remaining prospective progressor and control samples. Surviving SVM models comprised the tuberculosis risk signature, which computes a "tuberculosis risk score" based on blood gene expression levels measured at a single timepoint. The algorithm is fully described (appendix 1).

These analyses were executed using R or custom programs written in C++. Application of final signatures to predict tuberculosis risk was carried out using scripts written in R or an Excel spreadsheet (appendix 2). Statistical evaluation of prediction performance was done by analysis receiver operating characteristic curves (ROCs) using the R package pROC.[27]

Role of the funding source

The funders of this study had no role in study design, data collection, data analysis, data interpretation, or writing of the report. DEZ, AP-N, TJS, ET, LMA, AA, and WAH had full access to all the data in the study The corresponding author had full access to all the data in the study and had final responsibility for the decision to submit for publication.

Results

Between July 6, 2005, and April 23, 2007, we enrolled 6363 healthy adolescents from the ACS cohort; follow-up was completed by February, 2009 (appendix 1). Between Feb 27, 2005, and Dec 14, 2010, (South Africa) and between March 5, 2007, and Oct 21, 2010 (The Gambia), we enrolled 4466 healthy individuals from the GC6-74 cohort, of these 1197 were enrolled in the South African and 1948 enrolled in The Gambia (appendix 1).

46 ACS participants with microbiologically confirmed tuberculosis were identified as progressors (figure 1; appendix 2). For progressors, the time between sample collection and diagnosis with active tuberculosis ("time to diagnosis") ranged from 1 to 894 days (figure 2; appendix 2). 107 control participants who were infected with *M tuberculosis* at enrolment but who remained healthy during 2 years of follow-up were matched to progressors. Before analysis, progressors and controls were randomly divided into a training set of 37 progressors and 77 controls, and a test set of nine progressors and 30 controls (figures 1, 2).

Progressor and control participants in GC6-74 were household contacts of newly diagnosed index cases with pulmonary tuberculosis disease (figure 2). Two GC6-74 sites, South Africa and The Gambia, had sufficient numbers of progressors and controls to allow analysis, and were therefore included in this study (figures 1, 2). 43 progressors and 172 controls were identified at the South African site, whereas 30 progressors and 129 controls were identified at the Gambian site (figure 1; appendix 2).

RNA was isolated from all progressor and matched control samples of the ACS training set and analysed by RNA sequencing (figure 2; appendix 2). Data mining of the RNA sequencing data derived a candidate signature of risk for tuberculosis disease progression. The signature comprised paired splice junction data from 16 genes (appendices 1, 2). Expression of signature genes in samples from progressors increased as tuberculosis diagnosis approached (figure 3). Robust discrimination between progressors and controls based on the expression of the gene pairs in the signature was readily apparent (figure 3).

The predictive potential of the tuberculosis risk signature was shown within the ACS training set by cross validation (figure 2; appendix 1); the risk signature achieved 71·2% sensitivity in the 6 month period immediately before diagnosis, and 62·9% sensitivity 6–12 months before diagnosis, at a specificity of 80·6% (figure 3, table 1). During the 12–18 month period before diagnosis, the signature achieved 47·7% sensitivity.

To help with broad application, the tuberculosis risk signature was adapted to a practical platform, qRT-PCR (figure 2; appendix 2). The RNA sequencing and qRT-PCR versions of the tuberculosis risk signature were used to predict tuberculosis risk in the untouched ACS test set samples. This was done in a fully blinded manner, with all sample meta-data masked before making predictions. The ability of both versions of the signature to predict tuberculosis progression in healthy subjects was validated (figure 3, table 2).

	Platform	Days before tuberculosis diagnosis	ROC AUC (95% CI)	ROC p value	Sensitivity (95% CI)	Specificity (95% CI)	Threshold
ACS test							
All ACS test	RNA sequencing	..	0·69 (0·52–0·85)	0·018	41·7% (22·3–64·5)	89·9% (82·6–94·0)	82%
All ACS test	qRT-PCR	..	0·69 (0·54–0·85)	0·0095	46·7% (27·8–66·6)	90·9% (83·8–94·7)	76%
GC6-74							
All GC6-74	qRT-PCR	1–720	0·69 (0·63–0·76)	<0·0001	48·8% (39·9–57·7)	82·8% (78·7–86)	76%
South Africa	qRT-PCR	1–720	0·72 (0·63–0·81)	<0·0001	43·2% (31·7–55·5)	87·7% (82·7–91·2)	79%
The Gambia	qRT-PCR	1–720	0·67 (0·56–0·78)	0·001	50·0% (37·1–62·8)	81·9% (75·5–86·7)	78%
All GC6-74	qRT-PCR	1–360	0·72 (0·64–0·80)	<0·0001	53·7% (42·6–64·3)	82·8% (78·7–86·0)	76%
All GC6-74	qRT-PCR	361–720	0·65 (0·53–0·76)	0·0048	39·3% (25·8–54·8)	85·5% (81·7–88·5)	79%

ROC AUC=area under receiver operating characteristic curve. qRT-PCR=quantitative real-time PCR. GC6-74=Grand Challenges 6-74 study. ACS=adolescent cohort study.

Table 2: **Blind prediction performance of the tuberculosis risk signature on the ACS test set by RNA sequencing and qRT-PCR and on the GC6-74 cohorts from South Africa and The Gambia by qRT-PCR**

To determine whether inclusion of a larger number of genes would have increased accuracy of predictions, the performance of a random forest-based classifier comprised of 631 genes was assessed; the outcome was equivalent to when the tuberculosis risk signature was used for classification (appendices 1, 2).

For independent validation, the qRT-PCR-based signature was used to predict tuberculosis progression using samples collected from healthy participants in the GC6-74 adult household contact cohorts from South Africa and The Gambia (figure 2). Predictions were made in a blinded manner. The ability of the signature to predict tuberculosis progression in healthy participants were validated in these independent cohorts, irrespective of whether these were analysed individually or collectively ($p<0·0001$; figure 3, table 2). As for the ACS, the signature had greater sensitivity for predicting tuberculosis in samples collected closer to the time of diagnosis (figure 3, table 2).

Because the sensitivity of the tuberculosis risk signature increased as the time of diagnosis approached, we assessed performance of the risk signature for diagnosis of active tuberculosis disease. We did these analyses after adapting the signature to Illumina microarrays using data from the UK training cohort of Berry and colleagues (appendices 1, 2),[12] which enabled use of published datasets.[10–14] The signature readily differentiated active tuberculosis from latent infection in adult cohorts from the UK, South Africa, and Malawi, including in populations that were co-infected with HIV (appendices 1, 2). The signature also discriminated active tuberculosis from other pulmonary diseases (appendices 1, 2). Despite being derived from adolescents, the signature discriminated active, culture confirmed, tuberculosis from latent *M tuberculosis* infection and from other diseases in childhood (appendices 1, 2). Finally, applying the signature to data from a treatment study[10] showed that the active tuberculosis signature gradually disappears during 6 months of therapy (appendix 1, 2).

Discussion

Roughly one third of the world's population might harbour latent *M tuberculosis* infection and is at risk of active disease. We have identified a gene expression signature for predicting the risk of tuberculosis disease progression. This signature was discovered in a longitudinal analysis of South African adolescents with latent *M tuberculosis* infection who either developed tuberculosis disease or remained healthy. The signature was then validated in blinded samples from untouched adolescents of the same parent cohort. The signatures were again validated, in independent cohorts of longitudinally followed up household contacts of patients with tuberculosis disease from South Africa and The Gambia, who either developed tuberculosis disease or remained healthy. These results show that it is possible to predict progression from latent to active disease with whole blood gene expression measurements at any single timepoint up to 18 months before tuberculosis disease manifests.

The tuberculosis risk signature was discovered using RNA sequencing, a transcriptome analysis technology that is quantitative, sensitive, and unbiased.[28] The signature was formulated using a framework termed SVM, an extension of the k-TSP approach,[24] which robustly generates a tuberculosis risk score from gene expression data, with simple arithmetics (appendix 2). The signature was adapted from RNA sequencing to qRT-PCR, a more targeted and affordable technology. The power of the approach was shown by blinded validation of the qRT-PCR-based signature in the independent cohorts.

The tuberculosis risk signature predicted tuberculosis disease progression despite marked diversity between the ACS and GC6-74 cohorts. This result is encouraging in view of the different age ranges (adolescents *vs* adults), different infection or exposure status, distinct ethnic origin and genetic backgrounds,[29,30] differing local epidemiology,[1] and differing circulating strains of *M tuberculosis*[31] between South Africa and The Gambia. Distinct mechanisms of

progression might be elucidated when specific subgroups of progressors are analysed (eg, early *vs* late progressors in GC6-74). Targeted analyses to identify distinct mechanisms of progression are underway.

To explore potential application of the signature for targeted preventive therapy, we estimated the relative risk for tuberculosis disease between signature positive and negative people from a representative adult population from South Africa, where tuberculosis is endemic. The relative risk of tuberculosis disease is about 2 when IGRA or TST is used,[32] whereas the relative risk using our risk signature was between 6 and 14. Moreover, this risk signature would aid in detection of asymptomatic or undiagnosed tuberculosis disease, or both. For example, when applied to combined data from four studies of HIV-uninfected South African adults[10–13] involving 130 prevalent tuberculosis cases and 230 controls, the signature discriminated between patients with active tuberculosis and uninfected or healthy controls infected with *M tuberculosis* with 87% sensitivity and 97% specificity.

Although our focus was on prospective prediction of tuberculosis disease, we also showed that the risk signature was excellent for differentiating tuberculosis disease from latent infection and from other disease states. This ability to diagnose tuberculosis disease was not markedly affected by HIV status. The risk signature could also diagnose culture positive childhood tuberculosis, but not culture negative childhood disease.[33] These results suggest that the risk signature might represent bacterial load in the lung because culture positive childhood tuberculosis is likely associated with higher bacterial loads compared with culture negative disease. An association between the risk signature and bacterial load was further supported by meta-analysis of a published treatment study,[10] in which the signature relaxed during 6 months of antimicrobial therapy. It is presently not known whether the risk signature will be useful for predicting treatment failure or recurrence.

While enrichment analysis of published blood signatures for active tuberculosis implicates various biological processes in the disease, the sole gene module that was over-represented in the risk signature was interferon response (appendix 2). Overlap between 15 of the 16 genes in our prospective tuberculosis risk signature and the 393 gene signature of active tuberculosis disease from Berry and colleagues[12] suggests that chronic peripheral activation of the interferon response precedes the onset of active disease and the inflammatory manifestations of tuberculosis disease shown by previously published gene expression studies.[10–14] Although additional research is needed to understand the functional role of interferon responses during tuberculosis progression, pathogen induction of type I interferons and their detrimental effects on immunity to tuberculosis have been shown in several in-vivo studies in mice[34–36] and in-vitro experiments of human cells.[37] Nevertheless, not all interferon response genes in the risk signature might be associated with a poor outcome, since genes such as GBP1, STAT1, and TAP1 might have a protective role during tuberculosis infection (appendix 2).

Our predictive signature was obtained from transcriptomic analysis of peripheral whole blood. This compartment, although conveniently sampled, might not accurately represent pathogenic events in the lung, mainly affected by tuberculosis disease. Irrespective of this, circulating white blood cells can serve as sentinels of lung pathophysiology, as transcriptional changes occur when the cells migrate through this organ. To explore a possible cell-type specific origin of the risk signature, we used data from published global gene expression in whole blood and sorted peripheral blood mononuclear cells, monocytes, neutrophils, and T cells from healthy controls and patients with tuberculosis.[11] Differential expression of the risk signature genes between healthy controls and patients with tuberculosis was similar in whole blood and peripheral blood mononuclear cells (appendix 2), suggesting that contribution of granulocytes to the risk signature is redundant. Consistent with this hypothesis, differential expression of risk signature genes was apparent in monocytes and neutrophils. When compared to the diagnostic signature of Berry and colleagues,[12] reported to be derived from neutrophils, these results suggest that progression to active tuberculosis involves more diverse cell types.

So far, Sloot and colleagues[38] published the only report of prospective associations between blood gene expression and tuberculosis disease risk. With a predefined 141 gene panel, peripheral blood mononuclear cell RNA expression in 15 HIV-positive drug users who developed active tuberculosis disease was compared with 16 who did not develop tuberculosis. Four genes assayed showed nominal expression differences (unadjusted $p<0\cdot05$) and, when combined, two genes, *IL-13* and *AIRE*, fit the data (area under receiver operating characteristic curve fit=0·8). The association between these genes and tuberculosis progression was not validated in a test set or independent cohort; none of the four genes showed differences between progressors and controls in our whole blood RNA sequencing datasets.

Our results, showing that blood-based signatures in healthy individuals can predict progression to active tuberculosis disease, pave the way for the establishment of diagnostic methods that are scalable and inexpensive. An important first step would be to test whether the signature can predict tuberculosis disease in the general population, rather than the select populations included in this project; for example, the risk of tuberculosis disease in our populations was much higher than the lifetime risk of 10% encountered in the general population. The newly described signature holds potential for highly targeted preventive therapy, and therefore for interrupting the worldwide epidemic.

Contributors
HM, GDH, TH, SV, EJH, MO, RH, HMD, WHB, BT, HM-K, MH, SKP, SHEK, GW, and WAH designed the ACS and GC6-74 studies. AP-N, TJS, SSu, HM, ME, WW, DA, FK, TH, EJH, MO, RH, HMD, WHB, BT, HM-K, ACC, KD, MH, SKP, SHEK, GW, and WAH collected the data. DEZ, AP-N, TJS, ET, SSu, SSh, LMA, SHEK, AA, and WAH analysed the data and interpreted the results. DEZ, APN, TJS, ET, LMA, AA, and WAH had access to all of the data. DEZ, AP-N, TJS, ET, AA and WAH wrote the manuscript. All authors read and approved submission of the manuscript.

ACS cohort study group
South Africa: F Kafaar, L Workman, H Mulenga, T Scriba, R Ehrlich, D Abrahams, S Moyo, S Gelderbloem, M Tameris, H Geldenhuys, W Hanekom, G Hussey (South African Tuberculosis Vaccine Initiative, Institute of Infectious Disease and Molecular Medicine & Department of Paediatrics and Child Health, University of Cape Town, Cape Town); R Ehrlich (School of Public Health and Family Medicine, University of Cape Town, Cape Town). *Netherlands:* S Verver (KNCV Tuberculosis Foundation, The Hague, and Amsterdam Institute of Global Health and Development, Academic Medical Centre, Amsterdam). *USA:* Larry Geiter (Aeras, Rockville, MD).

The GC6-74 cohort study group
Germany: S H E Kaufmann (GC6-74 Principal Investigator), S K Parida, R Golinski, J Maertzdorf, J Weiner 3rd, M Jacobson (Department of Immunology, Max Planck Institute for Infection Biology, Berlin). *South Africa:* G Walzl, G Black, G van der Spuy, K Stanley, D Kriel, N Du Plessis, N Nene, A Loxton, N Chegou (DST/NRF Centre of Excellence for Biomedical TB Research and MRC Centre for TB Research, Division of Molecular Biology and Human Genetics, Stellenbosch University, Tygerberg); S Suliman, T Scriba, H Mahomed, J Hughes, K Downing, A Penn-Nicholson, H Mulenga, B Abel, M Bowmaker, B Kagina, W Kwong C, W Hanekom (South African Tuberculosis Vaccine Initiative, Institute of Infectious Disease and Molecular Medicine & Department of Paediatrics and Child Health, University of Cape Town, Cape Town). *Netherlands*: T H M Ottenhoff, M R Klein, M C Haks, K L Franken, A Geluk, K E van Meijgaarden, S A Joosten (Department of Infectious Diseases, Leiden University Medical Centre, Leiden); D van Baarle, F Miedema (University Medical Centre, Utrecht). *USA:* W H Boom, B Thiel (Tuberculosis Research Unit, Department of Medicine, Case Western Reserve University School of Medicine and University Hospitals Case Medical Center, Cleveland, Ohio); J Sadoff, D Sizemore, S Ramachandran, L Barker, M Brennan, F Weichold, S Muller, L Geiter (Aeras, Rockville, MD); G Schoolnik, G Dolganov, T Van (Department of Microbiology and Immunology, Stanford University, Stanford, California). *Uganda:* H Mayanja-Kizza, M Joloba, S Zalwango, M Nsereko, B Okwera, H Kisingo (Department of Medicine and Department of Microbiology, College of Health Sciences, Faculty of Medicine, Makerere University, Kampala). *UK:* H Dockrell, S Smith, P Gorak-Stolinska, Y-G Hur, M Lalor, J-S Lee (Department of Immunology and Infection, Faculty of Infectious and Tropical Diseases, London School of Hygiene & Tropical Medicine, London). *Malawi:* A C Crampin, N French, B Ngwira, A B Smith, K Watkins, L Ambrose, F Simukonda, H Mvula, F Chilongo, J Saul, K Branson (Karonga Prevention Study, Chilumba). *Ethiopia:* D Kassa, A Abebe, T Mesele, B Tegbaru (Ethiopian Health & Nutrition Research Institute, Addis Ababa); R Howe, A Mihret, A Aseffa, Y Bekele, R Iwnetu, M Tafesse, L Yamuah (Armauer Hansen Research Institute, Addis Ababa). *The Gambia:* M Ota, J Sutherland, P Hill, R Adegbola, T Corrah, M Antonio, T Togun, I Adetifa, S Donkor (Vaccines & Immunity Theme, Medical Research Council Unit, Fajara). *Denmark:* P Andersen, I Rosenkrands, M Doherty, K Weldingh (Department of Infectious Disease Immunology, Statens Serum Institute, Copenhagen).

Declaration of interests
We declare no competing interests.

Acknowledgments
This work was supported by grants from the Bill & Melinda Gates Foundation (BMGF) Global Health grants OPP1021972 and OPP1023483 and Grand Challenges in Global Health (GC6-74 grant 37772), the National Institutes of Health (RO1-AI087915), the European Union FP7 (ADITEC, 280873), and the Strategic Health Innovation Partnerships (SHIP) Unit of the South African Medical Research Council with funds received from the South African Department of Science and Technology. The ACS study was also supported by Aeras and BMGF GC 6-74 (grant 37772) and BMGF GC 12 (grant 37885) for QuantiFERON testing. We acknowledge European Commission (EC) FP7 NEWTBVAC contract number HEALTH.F3.2009 241745 and EC HOR2020 project TBVAC2020. AP-N and SSu were supported by Postdoctoral Research Awards from The Carnegie Corporation of New York. AP-N was also supported by The Claude Leon Foundation and the Columbia University-Southern African Fogarty AIDS International Training and Research Program (AITRP) through the Fogarty International Center, National Institutes of Health (grant # 5 D43 TW000231). SHEK also acknowledges support from European Union's Seventh Framework Programme (EU FP7) project ADITEC (HEALTH-F4-2011-280873; the Innovative Medicines Initiative Joint Undertaking "Biomarkers for Enhanced Vaccine Safety" project BioVacSafe (grant number 115308); and the European Union's Horizon 2020 project TBVAC2020 (grant number 643381).

References
1 WHO. Global Tuberculosis Report. 2014. http://www.who.int/tb/publications/global_report/en/ (accessed May 1, 2015).
2 Comstock GW, Livesay VT, Woolpert SF. The prognosis of a positive tuberculin reaction in childhood and adolescence. *Am J Epidemiol* 1974; **99:** 131–38.
3 Vynnycky E, Fine PE. Lifetime risks, incubation period, and serial interval of tuberculosis. *Am J Epidemiol* 2000; **152:** 247–63.
4 Shea KM, Kammerer JS, Winston CA, Navin TR, Horsburgh CR Jr. Estimated rate of reactivation of latent tuberculosis infection in the United States, overall and by population subgroup. *Am J Epidemiol* 2014; **179:** 216–25.
5 Horsburgh CR Jr, O'Donnell M, Chamblee S, et al. Revisiting rates of reactivation tuberculosis: a population-based approach. *Am J Respir Crit Care Med* 2010; **182:** 420–25.
6 Horsburgh CR Jr. Priorities for the treatment of latent tuberculosis infection in the United States. *N Engl J Med* 2004; **350:** 2060–67.
7 Wood R, Lawn SD, Caldwell J, Kaplan R, Middelkoop K, Bekker LG. Burden of new and recurrent tuberculosis in a major South African city stratified by age and HIV-status. *PLoS One* 2011; **6:** e25098.
8 Barry CE 3rd, Boshoff HI, Dartois V, et al. The spectrum of latent tuberculosis: rethinking the biology and intervention strategies. *Nat Rev Microbiol* 2009; **7:** 845–55.
9 Walzl G, Ronacher K, Hanekom W, Scriba TJ, Zumla A. Immunological biomarkers of tuberculosis. *Nat Rev Immunol* 2011; **11:** 343–54.
10 Bloom CI, Graham CM, Berry MP, et al. Detectable changes in the blood transcriptome are present after two weeks of antituberculosis therapy. *PLoS One* 2012; **7:** e46191.
11 Bloom CI, Graham CM, Berry MP, et al. Transcriptional blood signatures distinguish pulmonary tuberculosis, pulmonary sarcoidosis, pneumonias and lung cancers. *PLoS One* 2013; **8:** e70630.
12 Berry MP, Graham CM, McNab FW, et al. An interferon-inducible neutrophil-driven blood transcriptional signature in human tuberculosis. *Nature* 2010; **466:** 973–77.
13 Kaforou M, Wright VJ, Oni T, et al. Detection of tuberculosis in HIV-infected and -uninfected African adults using whole blood RNA expression signatures: a case-control study. *PLoS Med* 2013; **10:** e1001538.
14 Anderson ST, Kaforou M, Brent AJ, et al. Diagnosis of childhood tuberculosis and host RNA expression in Africa. *N Engl J Med* 2014; **370:** 1712–23.
15 Sutherland JS, Loxton AG, Haks MC, et al. Differential gene expression of activating Fcgamma receptor classifies active tuberculosis regardless of human immunodeficiency virus status or ethnicity. *Clin Microbiol Infect* 2014; **20:** O230–38.
16 Ottenhoff TH, Dass RH, Yang N, et al. Genome-wide expression profiling identifies type 1 interferon response pathways in active tuberculosis. *PLoS One* 2012; **7:** e45839.
17 Maertzdorf J, Weiner J 3rd, Mollenkopf HJ, et al. Common patterns and disease-related signatures in tuberculosis and sarcoidosis. *Proc Natl Acad Sci USA* 2012; **109:** 7853–58.
18 Maertzdorf J, Repsilber D, Parida SK, et al. Human gene expression profiles of susceptibility and resistance in tuberculosis. *Genes Immun* 2011; **12:** 15–22.

19 Maertzdorf J, Ota M, Repsilber D, et al. Functional correlations of pathogenesis-driven gene expression signatures in tuberculosis. *PLoS One* 2011; **6:** e26938.

20 Joosten SA, Fletcher HA, Ottenhoff TH. A helicopter perspective on TB biomarkers: pathway and process based analysis of gene expression data provides new insight into TB pathogenesis. *PLoS One* 2013; **8:** e73230.

21 Cliff JM, Lee JS, Constantinou N, et al. Distinct phases of blood gene expression pattern through tuberculosis treatment reflect modulation of the humoral immune response. *J InfectDis* 2013; **207:** 18–29.

22 SADOH. National tuberculosis management guidelines. 2014. http://www.tbonline.info/media/uploads/documents/ntcp_adult_tb-guidelines-27.5.2014.pdf (accessed May 1, 2015).

23 Wu TD, Nacu S. Fast and SNP-tolerant detection of complex variants and splicing in short reads. *Bioinformatics* 2010; **26:** 873–81.

24 Tan AC, Naiman DQ, Xu L, Winslow RL, Geman D. Simple decision rules for classifying human cancers from gene expression profiles. *Bioinformatics* 2005; **21:** 3896–904.

25 Bo T, Jonassen I. New feature subset selection procedures for classification of expression profiles. *Genome Biol* 2002; **3:** RESEARCH0017.

26 Shi P, Ray S, Zhu Q, Kon MA. Top scoring pairs for feature selection in machine learning and applications to cancer outcome prediction. *BMC Bioinformatics* 2011; **12:** 375.

27 Robin X, Turck N, Hainard A, et al. pROC: an open-source package for R and S+ to analyze and compare ROC curves. *BMC Bioinformatics 2011;* **12:** 77.

28 Wang Z, Gerstein M, Snyder M. RNA-Seq: a revolutionary tool for transcriptomics. *Nat Rev Genet* 2009; **10:** 57–63.

29 Tishkoff SA, Reed FA, Friedlaender FR, et al. The genetic structure and history of Africans and African Americans. *Science* 2009; **324:** 1035–44.

30 Black GF, Thiel BA, Ota MO, et al. Immunogenicity of novel DosR regulon-encoded candidate antigens of *Mycobacterium tuberculosis* in three high-burden populations in Africa. *Clin Vaccine Immunol* 2009; **16:** 1203–12.

31 Comas I, Coscolla M, Luo T, et al. Out-of-Africa migration and Neolithic coexpansion of *Mycobacterium tuberculosis* with modern humans. *Nat Genet* 2013; **45:** 1176–82.

32 Mahomed H, Ehrlich R, Hawkridge T, et al. TB incidence in an adolescent cohort in South Africa. *PLoS One* 2013; **8:** e59652.

33 Gray JW. Childhood tuberculosis and its early diagnosis. *Clin Biochem* 2004; **37:** 450–55.

34 Manca C, Tsenova L, Freeman S, et al. Hypervirulent M. tuberculosis W/Beijing strains upregulate type I IFNs and increase expression of negative regulators of the Jak-Stat pathway. *J Interferon Cytokine Res* 2005; **25:** 694–701.

35 Mayer-Barber KD, Andrade BB, Oland SD, et al. Host-directed therapy of tuberculosis based on interleukin-1 and type I interferon crosstalk. *Nature* 2014; **511:** 99-103.

36 Dorhoi A, Yeremeev V, Nouailles G, et al. Type I IFN signaling triggers immunopathology in tuberculosis-susceptible mice by modulating lung phagocyte dynamics. *Eur J Immunol* 2014; **44:** 2380–93.

37 Teles RM, Graeber TG, Krutzik SR, et al. Type I interferon suppresses type II interferon-triggered human anti-mycobacterial responses. *Science* 2013; **339:** 1448–53.

38 Sloot R, Schim van der Loeff MF, van Zwet EW, et al. Biomarkers can identify pulmonary tuberculosis in hiv-infected drug users months prior to clinical diagnosis. *EBioMedicine* 2015; **2:** 172–79.