AWARD NUMBER: W81XWH-18-1-0253

TITLE: A Rapid Blood Test to Differentiate Latent Tuberculosis from Active Disease

PRINCIPAL INVESTIGATOR: Antonino Catanzaro, MD

CONTRACTING ORGANIZATION: University of California, San Diego

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Fort Detrick, Maryland 21702-5012

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5c. PROGRAM ELEMENT NUMBER

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5d. PROJECT NUMBER

5e. TASK NUMBER

5f. WORK UNIT NUMBER

7. PERFORMING ORGANIZATION NAME(S) AND ADDRESS(ES)

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OFFICE OF CONTRACT & GRANT ADMINISTRATION
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8. PERFORMING ORGANIZATION REPORT NUMBER

9. SPONSORING / MONITORING AGENCY NAME(S) AND ADDRESS(ES)

U.S. Army Medical Research and Development Command
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14. ABSTRACT
The purpose of the study is to develop a blood-based TB test that meets or exceeds WHO Target Product Profiles for a rapid, biomarker-based, non-sputum triage test for detecting active TB disease. To accomplish this, the activities in Year 1 included improvements to the 3-gene mRNA signature, analysis of these improvements, prototype cartridge development, recruitment and blood collection in Moldova, and development of a secure data transmission system. Progress was made toward each of these goals, though validation of the improved signature and prototype cartridge are pending. Enrollment for Aim 2 is occurring at the expected rate. In Year 1 we developed a 9-gene signature that meets WHO TPP minimum criteria. Additional work will be done in Year 2 to improve on this signature and complete the prototype cartridge for validation and field testing.

15. SUBJECT TERMS
Tuberculosis, TB, mRNA signature, cartridge, triage test, blood test, finger stick, pre-clinical TB, active TB, latent TB, Moldova, WHO, TPP, biomarker-based

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1. INTRODUCTION:

The objective of this research is to develop a TB triage test which uses blood from a finger-stick that meets or exceeds WHO Targeted Product Profiles (TPP) for a rapid, biomarker-based, non-sputum triage test for detecting active TB. We plan to achieve this by developing a mRNA signature to discriminate patients with active TB from those with no TB, latent TB, or pre-clinical TB, validate this signature with prospectively collected blood from patients and contacts in the Republic of Moldova, transfer the signature into a Cepheid GeneXpert prototype cartridge, and then field test with a new cohort of prospectively enrolled patients suspected of having TB in the Republic of Moldova.

2. KEYWORDS:

Tuberculosis, TB, mRNA signature, cartridge, triage test, blood test, finger stick, pre-clinical TB, active TB, latent TB, Moldova, WHO, TPP, biomarker-based.

3. ACCOMPLISHMENTS:

What were the major goals of the project?

The major goal of the project is to develop a TB triage test using blood that meets or exceeds WHO Target Product Profiles for a rapid, biomarker-based, non-sputum triage test for detecting active TB disease (≥90% sensitivity when compared with the confirmatory test for active TB (both pulmonary and extrapulmonary) and ≥70% specificity against a microbiological reference standard. To accomplish this goal, our specific aims are 1) increase the power of our existing prototype mRNA signature to discriminate patients with active TB from those with no TB, latent TB or pre-clinical TB, 2) validate our improved TB signature using blood from 100 TB Index Cases and 450 household contacts in the Republic of Moldova and transfer the TB signature into the Cepheid GeneXpert prototype cartridge, and 3) field test the prototype cartridge with blood collected from 1000 patients in the Republic of Moldova who are suspected of having TB.

The approved SOW also states site-specific tasks (for UCSD, Moldova, Cepheid, Stanford, and the University of Arkansas) to meet these project goals:

All Sites: Scientific collaboration, data analysis
Site 1 University of California, San Diego: obtain local IRB/IACUC Approval (UCSD Phase 1 IRB), and HRPO approval, IRB Phase 2
Site 2 Phthisiopneumology Institute and Public Association Society of Clinical Mycobacteriology, Chisinau, Republic of Moldova: obtain local IRB/IACUC Approval (UCSD Phase 1 IRB), and HRPO approval, IRB Phase 2, enroll patients, collect blood, testing prototype cartridge in Moldova
Site 3 Cepheid: Perform multiplex RT-PCR, run PAXgene & finger stick protocol, validate cartridge using blood, finalize prototype cartridge
Site 4 Stanford University: Discover active & latent TB signatures, validate active & latent TB signatures
Site 5 University of Arkansas: Discover active & latent TB signatures, validate active & latent TB signatures.

What was accomplished under these goals?

Major activities: In Year 1 the major activities included scientific collaboration, discovery and augmentation of the 3-gene signature, initiation of enrollment and blood collection in Moldova for validation of the TB signature, and progress toward prototype cartridge development:

Scientific collaboration. In Year 1 of the project, UCSD executed subcontracts with each of the sites. Once per quarter, all sites came together for an “All-Hands” call to discuss progress and barriers. A weekly webinar between the PI and the Moldova site occurred throughout Year 1. These calls consisted of discussions of local TB control practices, and planning for participant recruitment, data collection, procuring laboratory supplies, and sample shipment. Each of the quarterly and weekly calls contributed to greater understanding and scientific collaboration between sites.

Discovery and augmentation of the 3-gene signature. In Year 1, Stanford completed in silico work to develop a more complex mRNA signature of 9 genes. This 9-gene signature met and exceeded minimum WHO TPP criteria as well as optimum criteria (95% sensitivity, 80% specificity) for distinguishing patients with active TB disease from healthy controls (observed performance 95% sensitivity, 83% specificity). The work that occurred in Year 1 to support this outcome involved identification and curation of gene expression datasets that profiled samples from various bacterial and viral infections and other lung diseases. The Stanford collaborators then created a database of these gene expression datasets and compared 16 gene signatures for TB diagnosis across more

than 20 datasets. This involved analysis of 7,254 samples (healthy, latent TB infection, other diseases, and active TB (ATB) in three iterative stages: discovery, hold-out validation, and independent validation. The discovery/hold-out validation phase was used to see which signature responded best. Once locked, the independent validation took place on a new dataset. This approach is much like the one used to develop the 3-gene signature but with significantly more data. Hundreds of genes were narrowed down to the 9-gene signature by examining performance to discriminate 22 groups of other conditions. Data were compared to the 3-gene signature to analyze improvements of the 9-gene signature. Although the 9-gene signature met TPP criteria for distinguishing ATB from heathy controls, the breakdown of ATB compared to latent TB and other diseases needs further improvement of specificity: when held at a 95% sensitivity, the specificity for detecting ATB against healthy controls, latent TB infection, and other diseases was 75%, 57%, and 64%, respectively.

The breakdowns for the key outcomes are provided in summary tables:

Comparison	Specificity at 90% sensitivity		
	Discovery	Hold-out validation	Independent validation
ATB vs Healthy	85	90	88
ATB vs LTBI	81	76	73
ATB vs other diseases	60	57	76

Comparison	Sensitivity at 90% specificity		
	Discovery	Hold-out validation	Independent validation
ATB vs Healthy	87	90	89
ATB vs LTBI	87	80	80
ATB vs other diseases	57	55	74

Comparison	Specificity at 95% sensitivity		
	Discovery	Hold-out validation	Independent validation
ATB vs Healthy	61	77	75
ATB vs LTBI	59	66	57
ATB vs other diseases	42	49	64

Comparison	Sensitivity at 95% specificity		
	Discovery	Hold-out validation	Independent validation
ATB vs Healthy	81	85	83
ATB vs LTBI	79	80	68
ATB vs other diseases	37	35	54

Number of samples	Discovery	Hold-out validation	Independent validation	Total samples
Healthy	994	399	497	1890
LTBI	99	41	324	464
Other diseases	1405	504	2098	4007
ATB	285	119	489	893
Total				**7254**

Stated goals not met: validation of the active and latent TB score were not complete as of the end of Year 1.

It was determined in Year 1 that Cepheid could not create a cartridge for a 9-gene signature. Cepheid began work on specifying the parameters a signature must meet in order for an RNA-signature cartridge to be feasible within the study timeframe (discussed in more detail in the section 'Changes in approach and reasons for change'). For these reasons, at the end of Year 1, Stanford began work towards a sub-9-gene signature. Plans for the next reporting period are discussed in the appropriate section of this report.

Initiation of enrollment and blood collection in Moldova for validation of the TB signature. Shortly after funding was awarded, the DoD IRB review process was initiated, and approval was granted on March 2, 2019. After final logistics were put in place, enrollment began in Moldova in April 2019, month 7 of the project. At the end of Year 1, 39 index cases and 293 close contacts had been enrolled (see Figure 1 and 2) out of the expected 100 index cases and 450 contacts to be enrolled by the end of Year 2. Current enrollment averages project completion of enrollment sooner than anticipated, though we continue to actively monitor for any changes that could hinder this progress. We believe this success is linked to the harmonization with the NIH protocol. In Year 1 the University of Arkansas (UARK) data core was set up using paper forms to record data, a scanner which automatically scans into a secure server and the software REDCap has been installed to house project data. A high-speed scanner was sent to Moldova and configured for secure transmission of case report forms (CRFs) to the server in Arkansas.

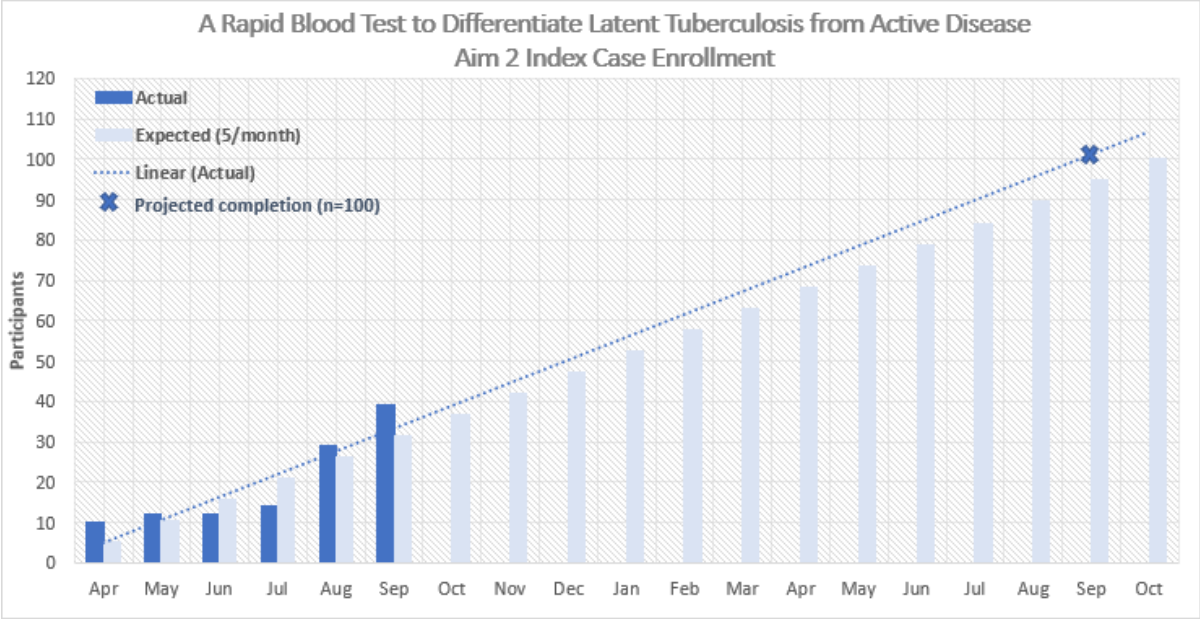


Figure 1 Index Case enrollment for Aim 2: validation of signature

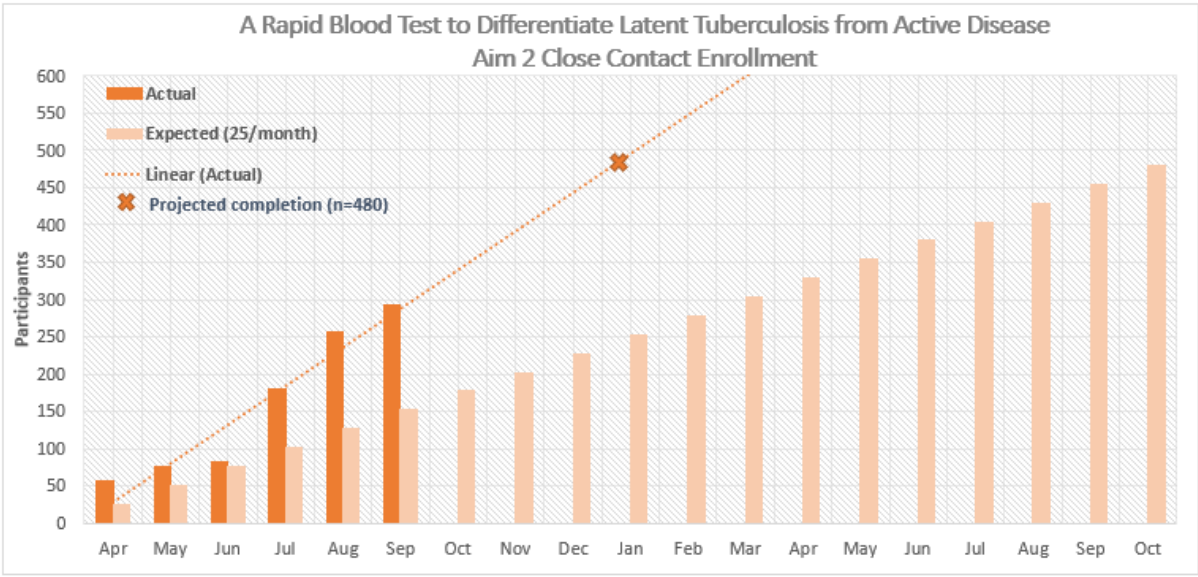


Figure 2 Close Contact enrollment for Aim 2: validation of signature

Progress toward prototype antigen-stimulated ("Robal") cartridge development. During Year 1 Cepheid began work on the RNA signature cartridge. Due to discoveries in the Stanford work and the availability of comparator data in Moldova (discussed in actual or anticipated problems), Cepheid focused largely on the proposed alternative hypothesis to develop an antigen-stimulated cartridge. Cepheid has provided these specific updates on their progress:

- **Prototype GeneXpert cartridge assay development work for antigen-stimulated blood**
  - This was started during autumn 2018 (funded by Cepheid), and was during Q3-Q4 pursued according to a simplified version of Cepheid's standard procedure used during Concept Phase and Technical Feasibility Phase. The two prototype cartridges (four markers plus controls each) are "open" such that they can be run with liquid PCR reagents ("wet" master mix format), as well as with lyophilized reagent beads (more similar to a final Cepheid product). The eight markers in the two cartridges are the most promising subset of a 16-gene candidate set.
  - The specific objective was optimization of the two prototype GeneXpert cartridges so that the laborious manual procedure of mRNA isolation, cDNA synthesis and -dilution, followed by parallel qPCRs can later on be replaced by a cartridge-based analysis approach for the large number of blood samples to be collected in Moldova within this project.
  - For optimization purposes, blood from healthy blood donors was used, after Mitogen stimulation and lysis/stabilization with different GeneXpert compatible buffer candidates.

The first set of samples from Moldova was received by Cepheid at the end of Year 1. Both PAXgene tubes (n=280) and QFT lysates (n=305) were sent. QFT lysates were prioritized for the enrolled participants as these samples are time-sensitive and were given priority in the available space in this first shipment; remaining PAXgene tubes will be sent with future shipments. UCSD and Cepheid agreed to and organized scheduled monthly shipments from Moldova to Cepheid going forward to ensure RNA quality Cepheid has provided these specific updates:

- **Evaluation on a small number of clinical samples**
    - Since no blood samples was obtained from Moldova on time to evaluate the prototype cartridges during Year 1 of this project, the "Q3 prototype GeneXpert cartridges" were evaluated using prospectively collected blood from another collaboration (funded by Cepheid).
    - The performance of the prototype GeneXpert cartridges as compared to the manual procedure (mRNA isolation, cDNA synthesis and -dilution, followed by qPCRs).
    - During Q4, we completed the comparative analysis of all GeneXpert- and manual procedure data for the Cepheid-funded clinical samples.
    - Since performance characteristics did not fully meet Cepheid's and the project-specific specs, we initiated a new round of continued optimization during Q4.
    - This empirical lab work has started and will continue during Year 2
        - Iterative oligonucleotide re-design and testing by using bioinformatics and wet-lab (ongoing)
        - Re-optimization of concentrations of critical qRT-PCR reagents
        - Further optimization of GeneXpert in-cartridge RNA isolation

Specific objectives: the major activities in Year 1 supported the specific objectives outlined in the SOW for Year 1 including: discovery and augmentation of 3-gene TB signature, enrollment and blood collection in Moldova, prototype cartridge development, and scientific collaboration.

Significant results or key outcomes: In Year 1 a significant accomplishment was completion of analysis for improvement of the 3-gene signature and development of a 9-gene signature. The results for the 9-gene signature from the 7,254 samples were significant toward planning for an improved signature that meets WHO TPP minimums (see Summary Tables, page 5).

What opportunities for training and professional development has the project provided?

Nothing to report.

How were the results disseminated to communities of interest?

Nothing to report.

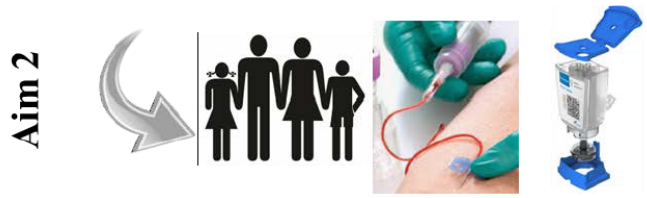
What do you plan to do during the next reporting period to accomplish the goals?

In the next immediate reporting period (Quarter 1 Year 2) we plan to execute Year 2 subcontracts with each of the sites and continue our collaboration on regular schedule as we did in Year 1. We will also continue enrollment and data collection activities in Moldova and monitor enrollment for any changes from the projected goals. The UARK site will develop an automated system which process scans via Optical Character/Mark Recognition to transform scans into data that is uploaded into REDCap. They also plan to send data collected in Moldova to Cepheid to validate the antigen-based cartridge.

In the next annual reporting period (Year 2), to build on the work that has been completed at Stanford in Year 1, we will continue to collaborate on the optimization of the gene signature. The activities for the next reporting period come from from decisions described in detail in section 5 of this report (changes in approach). Specifically, the Stanford site will use the in silico methodology to derive an mRNA signature with less than 9 genes (sub 9 gene signature) that meets or exceeds additional criteria provided by Cepheid regarding minimum acceptable improvements to performance and improved compatibility with technical specifications of their cartridge system. They will conduct in silico performance analysis of the existing 3-gene, 9-gene, and sub-9-gene signatures and determine additive value of each gene in the final multi-gene signature. We will also conduct empiric NanoString evaluations of blood samples collected in Moldova and, using this technology, will explore the empiric performance of each signature and the additive value of each component of the final signature. To accomplish this, UCSD will coordinate the delivery of PAXgene tubes collected in Moldova in Year 1 to the Stanford site for analysis. To support this work, Cepheid will provide specifications to Stanford for maximizing compatibility of the signature with the prototype assay. While the signature is finalized at Stanford and the patent is filed, Cepheid will continue to pursue the antigen stimulated prototype cartridge work as an alternative strategy. Cepheid has provided these additional updates:

Plan for Q1 Year 2

- Now as patient enrollment with blood shipments to Cepheid is established, we'll focus on Aim 2 of the Proposal:



Validate our improved TB signature using blood from 100 TB *Index Cases* & 450 household contacts in the Republic of Moldova. Transfer TB signature into Cepheid GeneXpert prototype cartridge.

- For marker/signature validation at Cepheid, Solna, Sweden, mRNA will be isolated from the 9 ml stabilized blood samples using Roche mRNA Isolation Kit for Blood and Bone Marrow (Roche) according to the Package Insert. The isolated mRNAs will be directly subjected to cDNA synthesis using Reverse Transcription System (Promega). The cDNAs will then be diluted and stored at -20°C prior to qPCR.
- The qPCR analysis will be done in single plex PCR, 96-well format using a BioRad real-time PCR plate instrument, with approx. 16 qPCR assays designed and developed at Cepheid, Solna, Sweden, measuring the approx. 16 candidate markers and reference genes. All primers and probes will be synthesized by Cepheid's DNA and protein chemistry specialists at our manufacturing site in Bothell, Washington, USA and contain Cepheid's proprietary dyes, quenchers, and modified bases, to enable a smooth transformation into the GeneXpert cartridge format.
- When markers/signatures have been validated using patient blood from Moldova (Approx. Q2 Year 2), the two prototype cartridges will be merged into one, containing the final marker signatures.

4. IMPACT:

What was the impact on the development of the principal discipline(s) of the project?

The principle discipline of the project is the quantitative biology of mRNA host immune biomarkers, and the potential of these markers to predict active TB disease and to distinguish active TB disease from latent infection and pre-clinical TB disease in order drive innovative diagnostics development. In this context, the first year of the study was a major success. Stanford investigator on the project, Dr. Khatri, used novel "big data" techniques to mine existing data sets in order to discover unique mRNA combinations (signatures) that had improved ability to distinguish active TB from other disease states. Prior to this project, Dr Khatri had demonstrated that a unique 3-gene mRNA signature could almost meet the minimal diagnostic performance criteria required for triaging patients at risk for TB (sensitivity 90%, specificity 70%). During this study period he has shown in silico that a more complex mRNA signature of 9 genes could not only meet and exceed minimum criteria but also meet optimum criteria (95% sensitivity, 80% specificity) for distinguishing patients with active TB disease from healthy controls (observed performance 95% sensitivity, 83% specificity). The performance distinguishing active TB from other disease groups was less successful (observed performance 95% sensitivity, 54% specificity). The importance of this set of patients is not clear at this point.

There is more work to do to improve performance still further while reducing the complexity of the mRNA signature to reduce the cost of translating the signature into a working prototype diagnostic device, it is important to recognize how significant this is for the field of biomarker-based TB diagnostics as there currently no equivalent biomarker signature with similar performance that can be maintained across a broad range of clinical patients from many parts of the world.

What was the impact on other disciplines?

The novel data analysis and in silico techniques utilized by the Stanford group to identify this novel biomarker signature is a generalizable method that will yield significant diagnostic benefits beyond the field of TB research. These methods can be applied similarly to data sets from cancer patients and those infected with other infectious diseases in order to yield predictive mRNA signatures that could not only shed light on the basic biology of disease processes in vivo, but could also become the foundation of novel diagnostic approaches to identify critical diseases earlier in order to improve interventions and treatment outcomes.

What was the impact on technology transfer?

This project did two things to impact future commercial technologies in the past year of project activities.

1. Stanford identified a high-performance mRNA signature in silico that has the potential to meet both minimum and

optimal criteria for differentiating patients with TB disease from healthy controls. This signature is currently undergoing patent registration at Stanford in order to prepare for potential licensing and transfer to project diagnostic partner, Cepheid, in order for Cepheid to conduct further validation studies.
2. This project also funded the collection of unique clinical samples and data from TB patients and their family contacts in Moldova, which provide a unique resource for validating early biomarker validation studies. These samples and data were transferred to Cepheid in order to help validate an early prototype antigen stimulated ("Robal") cartridge that Cepheid has developed to test a biomarker signature designed to distinguish patients with active TB from healthy controls and those with latent TB. These samples reduce a significant obstacle in the development pathway for Cepheid.

What was the impact on society beyond science and technology?

Our project activities impact beyond science and technology were limited in year 1. Mostly they were confined to what we as project lead learned about the complex collaborative process needed to bring lead scientists, experienced technicians and corporate executives together in order to keep an industry diagnostic development process moving forward. It is a complex balance of the value of anticipated gains from new discoveries against the significant costs of research and development to implement those gains into a prototype device. This first year gave us new insights into this process and partners which will serve us well in the remainder of the project as move forward with development.

5. CHANGES/PROBLEMS:

Changes in approach and reasons for change

We are exploring the possibility of a *sub*-9-gene signature. During Year 1, Cepheid stated that they needed a signature with less than 9 genes (*sub*-9 gene signature) as the time remaining in this project precludes production of a cartridge that can evaluate a 9-genes. Cepheid is clarifying the specifications for a *sub*-9-gene signature cartridge. The Stanford is analyzing the data to inform a decision on the genes that would be included in the revised sub-9-gene signature. An all-hands call is planned to discuss the specific approach following these analyses in November 2019. Though this is not an actual change in the approach specified in the study aims, it is relevant to this reporting period and may dictate decision making in future steps. The work on the alternative approach of an antigen-based cartridge is still moving forward within the study timeframe and currently there is no planned change in approach to this.

Actual or anticipated problems or delays and actions or plans to resolve them

As described earlier, during Year 1 Stanford worked on in silico development of an improved mRNA signature. A delay that was discovered was a requirement by Stanford University to acquire intellectual property (IP) protection for the signature prior to dissemination to Cepheid. We have already taken steps to resolve this: the Stanford site PI, Dr. Khatri, has requested and received approval for expedited IP processing once the signature is complete.

Another delay that occurred was procurement of the ELISA kits needed to run the QuantiFERON (QFT) in Moldova, preventing the data from being shared with Cepheid for validation of the latent TB prototype cartridge. We learned this was due to significant delays by the supplier in Romania requiring supplemental documentation to support the quantity of kits ordered, as well as the kits being on backorder once the order was successful. To resolve this, Cepheid has offered to send ELISA kits to Moldova, though as of the end of Year 1 this was not yet completed. As soon as the kits are received by the supplier, the Moldova site will place the next order to help prevent this delay from occurring again.

There has been some delay in establishing an efficient data pipeline from Moldova to the data management center at UARK, and subsequently to collaborators. This has resulted in delays providing clinical data to Cepheid for marker/signature validation. To mitigate this, we plan to discuss logistics for a regular data delivery schedule for both clinical and QFT-plus data following the sample shipment, subject to availability of raw data from Moldova. This has been successfully executed for regular sample shipments, so we anticipate setting up a similar structure for data is possible. Additionally, the UARK team is actively working on improvements to the flow of data from Moldova such as establishment of a high-speed scanner, development of a data reconciliation report, and more regular interactions with the clinical team in Moldova. In the long term, the UARK team plans to implement a secure website where collaborators can view live updates to database variables relevant to their site, which we anticipate will improve the flow of information across all collaborating partners.

Changes that had a significant impact on expenditures

The process of establishing sub-awards for our four collaborating sites took significantly longer than anticipated

(between 8 and 11 months after the project began). These delays were primarily due to extended contract negotiations between UCSD and our collaborators regarding Intellectual Property (IP) issues, as well as to the development of detailed Statements of Work for deliverables from each site. As a result, the sites were not able to submit many invoices prior to the end of the first project year, and though the sub-award funds have been liened, the Actual Expenditures in the sub-award category are well below the Projected Expenditures in this same category.

Fortunately, all sub-awards have now been established, and some invoices have been received. We anticipate no further delays in expenditures from any of our collaborating sites.

Significant changes in use or care of human subjects, vertebrate animals, biohazards, and/or select agents

No significant deviations, unexpected outcomes, or changes in approved protocols for the use or care of human subjects, vertebrate animals, biohazards, and/or select agents occurred during the reporting period. The DoD Institutional Review Board approved this project for enrollment of human subjects on March 2, 2019 and no amendments were made since this this date. No enrollment occurred before the approval date.

Significant changes in use or care of human subjects
Nothing to report.

Significant changes in use or care of vertebrate animals
Nothing to report.

Significant changes in use of biohazards and/or select agents
Nothing to report.

6. PRODUCTS:

Publications, conference papers, and presentations

Hayley Warsinske, Rohit Vashisht, Purvesh Khatri. Host-response-based gene signatures for tuberculosis diagnosis: A systematic comparison of 16 signatures. PLoS Medicine 2019, 16(4):e1002786.

Website(s) or other Internet site(s)

Nothing to report.

Technologies or techniques

Nothing to report.

Inventions, patent applications, and/or licenses

Nothing to report.

Other Products

Nothing to report.

7. PARTICIPANTS & OTHER COLLABORATING ORGANIZATIONS

What individuals have worked on the project?

Name:	Antonino Catanzaro, MD
Project Role:	Project PI
Nearest person month worked:	2
Contribution to Project:	No change

Name:	Timothy Rodwell
Project Role:	UCSD Co-Investigator
Nearest person month worked:	1
Contribution to Project:	No change

Name: Peter Chiles
Project Role: Laboratory Manager
Nearest person month worked: 1
Contribution to Project: No change

Name: Laura Myhovich
Project Role: Project Coordinator
Nearest person month worked: 1
Contribution to Project: No change

Name: Malin Nygren
Project Role: Cepheid Site PI
Nearest person month worked: 1 (funded by Cepheid)
Contribution to Project: Project management

Name: Jennie Hermansson
Project Role: Research Scientist, Cepheid
Nearest person month worked: 11
Contribution to Project: No change

Name: Raquel Rodrigues Palla
Project Role: Research Scientist, Cepheid
Nearest person month worked: 11
Contribution to Project: No change

Name: Sarah Tidström
Project Role: Research Scientist, Cepheid
eRA Commons ID: n/a
Nearest person month worked: 1
Contribution to Project: No change

Name: Purvesh Khatri
Project Role: Stanford Site PI
Nearest person month worked: 3
Contribution to Project: No change

Name: Rohit Vashisht
Project Role: Research Scientist, Stanford
Nearest person month worked: 3
Contribution to Project: No change

Name: Michele Donato
Project Role: Postdoc, Stanford
Nearest person month worked: 3
Contribution to Project: No change

Name: Aditya Rao
Project Role: Graduate student, Stanford
Nearest person month worked: 4
Contribution to Project: No change

Name: Hayley Warsinske
Project Role: Postdoc, Stanford
Nearest person month worked: 12
Contribution to Project: Database creation

Name: Madeleine Scott
Project Role: Graduate student, Stanford
Nearest person month worked: 3
Contribution to Project: Dataset curation

Name: Donald Catanzaro
Project Role: University of Arkansas Site PI
Nearest person month worked: 6
Contribution to Project: No change

Name: Ahla Ko
Project Role: Graduate Student, University of Arkansas
Nearest person month worked: 12
Contribution to Project: Data system development

Name: Valeriu Crudu
Project Role: Moldova Site PI
Nearest person month worked: 1
Contribution to Project: No change

Name: Elena Tudor
Project Role: Moldova Clinical Coordinator
Nearest person month worked: 4
Contribution to Project: No change

Name: Nelly Ciobanu
Project Role: Moldova Laboratory Coordinator
Nearest person month worked: 6
Contribution to Project: No change

Has there been a change in the active other support of the PD/PI(s) or senior/key personnel since the last reporting period?

Nothing to report.

What other organizations were involved as partners?

Organization Name: **University of Arkansas**
Location of Organization: Fayetteville, Arkansas
Partner's contribution to project:
a) Facilities (PI office space, data core facilities)
b) Collaboration

Organization Name: **Stanford University**
Location of Organization: Stanford, California
Partner's contribution to project:
a) Facilities (PI office space, computational biology and translational medicine research laboratory space)
b) Collaboration

Organization Name: **Cepheid**
Location of Organization: Solna, Sweden
Partner's contribution to project:
a) In-kind support (PI salary paid by Cepheid)
b) Facilities (PI office space, R&D and manufacturing facilities)
c) Collaboration

Organization Name: **Institute of Phthisiopneumology**
Location of Organization: Chisinau, Moldova
Partner's contribution to project:
a) Facilities (PI office space, Microbiology & Morphology laboratory)
b) Collaboration

Organization Name: **Public Association Society of Clinical Mycobacteriology from Republic of Moldova**
Location of Organization: Chisinau, Moldova
Partner's contribution to project:
a) Collaboration

8. SPECIAL REPORTING REQUIREMENTS

Award Chart (Page 11) and Quad Chart (Page 12)

9. APPENDIX

PLoS journal article (Pages 13-31)

PR171076: A Rapid Blood Test to Differentiate Latent Tuberculosis from Active Disease

PI: Antonino Catanzaro; University of California, San Diego; California **Budget:** $3,406,602

Topic Area: PRMRP-TTDA **Mechanism:** W81XWH-17-PRMRP-TTDA

Research Area(s): Tuberculosis **Award Status:** September 30, 2018 – September 29, 2021

Study Goals:

The major goal of the project is to develop a TB triage test using blood that meets or exceeds WHO Target Product Profiles for a rapid, biomarker-based, non-sputum triage test for detecting active TB disease (≥90% sensitivity when compared with the confirmatory test for active TB (both pulmonary and extrapulmonary) and ≥70% specificity against a microbiological reference standard.

Specific Aims:

1) Use bioinformatics on our database of RNA expression to select genes which increase the robustness and performance of our 3-gene signature to discriminate active TB, pre-clinical TB, and healthy, uninfected individuals
2) Validate our TB signature using blood from TB index cases & their contacts in the Republic of Moldova; transfer the TB signature into the Cepheid GeneXpert prototype cartridge
3) Field test the prototype cartridge using blood collected from 1,000 individuals

Key Accomplishments and Outcomes:

Publications: Hayley Warsinske, Rohit Vashisht, Purvesh Khatri. Host-response-based gene signatures for tuberculosis diagnosis: a systematic comparison of 15 signatures. PLoS Medicine 2019, 16(4):e1002786.

Patents: none to date

Funding Obtained: $3,406,602

A Rapid Blood Test to Differentiate Latent Tuberculosis from Active Disease

PR171076

W81XWH1810253

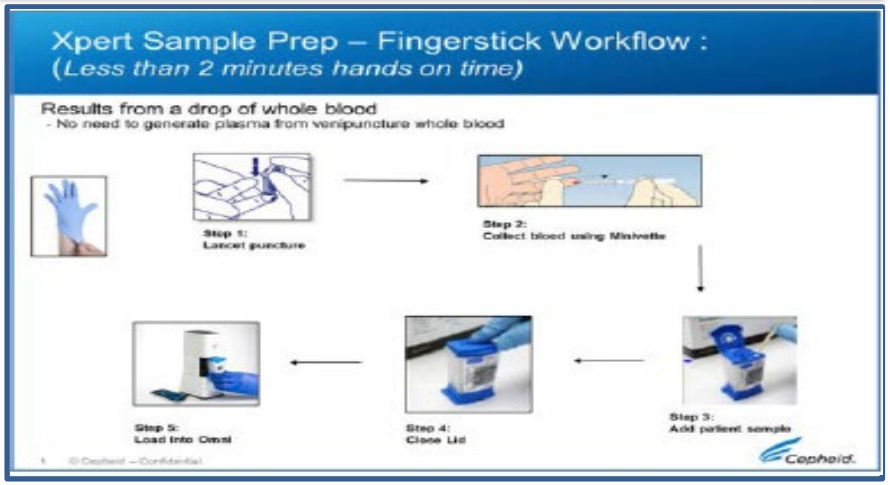
PI: Antonino Catanzaro, MD **Org:** The Regents of the University of California, San Diego **Award Amount:** $3,406,602

Study/Product Aim(s)

1) Use bioinformatics on our database of RNA expression to select genes which increase the robustness and performance of our 3-gene signature to discriminate active TB, pre-clinical TB, and healthy, uninfected individuals
2) Validate our TB signature using blood from TB index cases & their contacts in the Republic of Moldova; transfer the TB signature into the Cepheid GeneXpert prototype cartridge
3) Field test the prototype cartridge using blood collected from 1,000 individuals

Approach

For Aim 1, we will apply our computational framework for integrated multi-cohort analysis of gene expression data to pre-collected datasets (which include profiled patients with latent Mtb infection, along with healthy controls, and patients with active TB or other diseases), utilizing the WHO TB Diagnostics Development framework for test development. In Aims 2 & 3, we will recruit TB Index Cases from the Republic of Moldova. Nurses will conduct epidemiological contact investigations to identify transmissions of TB to a close contact. Bloods will be collected and tested, first for improvement of the prototype (Aim 2), then for cartridge validation (Aim 3).



Accomplishments: Established final subaward, continued recruitment of index patients and close contacts in Moldova, performed in-silico analysis of the 9-gene host response-based biomarker signature, and progressed toward the development of an antigen-stimulated prototype cartridge.

Activities CY	18	19	20	21
1. Discovery & Augmentation of 3-gene TB signature				
2. Validation				
3. Enrollment & blood collection in Moldova				
4. Prototype cartridge development				
5. Field Trial				
Estimated Budget ($K)	56,410	1,373,446	1,122,801	853,945

Goals/Milestones

CY18 Goal – Project Initiation & Study Partner Engagement

☑Scientific collaboration

CY19 Goals

Discovery/Augmentation/Validation of 3-gene TB signature

☐Discovery & Validation of Active & Latent TB Scores

Enrollment & Blood Collection in Moldova

☑Obtain local IRB/IUCAC approval (IRB Phase 1) & HRPO approval

☑Enroll patients, collect blood

CY20 Goal – Prototype Cartridge Development

☐Perform RT-PCR; Run PAXgene & finger-stick protocol

☐Validate cartridge

CY21 Goal – Field Trial

☐Finalize prototype cartridge; field test in Moldova

Comments/Challenges/Issues/Concerns: All subawards finalized; subaward liens as of 10/29/19 = $743,411 remaining to be invoiced for Project Year 1/not included in Actual Expenditure amount below

Budget Expenditure to Date

Projected Expenditure: $1,086,945

Actual Expenditure: $243,149

Updated: 10/29/2019

PLOS MEDICINE

RESEARCH ARTICLE

Host-response-based gene signatures for tuberculosis diagnosis: A systematic comparison of 16 signatures

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Data Availability Statement: Data are available from the NCBI GEO at: https://www.ncbi.nlm.nih.gov/geo/ The accession numbers for the individual studies are listed in Table 2.

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Abstract

Background

The World Health Organization (WHO) and Foundation for Innovative New Diagnostics (FIND) have published target product profiles (TPPs) calling for non-sputum-based diagnostic tests for the diagnosis of active tuberculosis (ATB) disease and for predicting the progression from latent tuberculosis infection (LTBI) to ATB. A large number of host-derived blood-based gene-expression biomarkers for diagnosis of patients with ATB have been proposed to date, but none have been implemented in clinical settings. The focus of this study is to directly compare published gene signatures for diagnosis of patients with ATB across a large, diverse list of publicly available gene expression datasets, and evaluate their performance against the WHO/FIND TPPs.

Methods and findings

We searched PubMed, Gene Expression Omnibus (GEO), and ArrayExpress in June 2018. We included all studies irrespective of study design and enrollment criteria. We found 16 gene signatures for the diagnosis of ATB compared to other clinical conditions in PubMed. For each signature, we implemented a classification model as described in the corresponding original publication of the signature. We identified 24 datasets containing 3,083 transcriptome profiles from whole blood or peripheral blood mononuclear cell samples of healthy controls or patients with ATB, LTBI, or other diseases from 14 countries in GEO. Using these datasets, we calculated weighted mean area under the receiver operating characteristic curve (AUROC), specificity at 90% sensitivity, and negative predictive value (NPV) for each gene signature across all datasets. We also compared the diagnostic odds ratio (DOR), heterogeneity in DOR, and false positive rate (FPR) for each signature using bivariate meta-analysis. Across 9 datasets of patients with culture-confirmed diagnosis of ATB, 11 signatures had weighted mean AUROC > 0.8, and 2 signatures had weighted mean AUROC $\leq$ 0.6. All but 2 signatures had high NPV (>98% at 2% prevalence). Two gene signatures achieved the minimal WHO TPP for a non-sputum-based triage test. When including datasets with clinical diagnosis of ATB, there was minimal reduction in the

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Competing interests: I have read the journal's policy and the authors of this manuscript have the following competing interests: PK is a co-founder of and a scientific advisor to Inflammatix, Inc. Inflammatix played no role in this manuscript. PK is an inventor on the Sweeney3 signature pending patent owned by Stanford University, which has been licensed for commercialization.

Abbreviations: ACS, Adolescent Cohort Study; ATB, active tuberculosis; AUROC, area under the receiver operating characteristic curve; DOR, diagnostic odds ratio; EBI, European Bioinformatics Institute; FIND, Foundation for Innovative New Diagnostics; FPR, false positive rate; GEO, Gene Expression Omnibus; IGRA, Interferon Gamma Release Assay; LTBI, latent tuberculosis infection; Mtb, Mycobacterium tuberculosis; NCBI, National Center for Biotechnology Information; NPV, negative predictive value; OD, other disease; PBMC, peripheral blood mononuclear cell; POC, point of care; PPV, positive predictive value; TB, tuberculosis; TPP, target product profile; TST, tuberculin skin test; WHO, World Health Organization.

weighted mean AUROC and specificity of all but 3 signatures compared to when using only culture-confirmed ATB data. Only 4 signatures had homogeneous DOR and lower FPR when datasets with clinical diagnosis of ATB were included; other signatures either had heterogeneous DOR or higher FPR or both. Finally, 7 of 16 gene signatures predicted progression from LTBI to ATB 6 months prior to sputum conversion with positive predictive value > 6% at 2% prevalence. Our analyses may have under- or overestimated the performance of certain ATB diagnostic signatures because our implementation may be different from the published models for those signatures. We re-implemented published models because the exact models were not publicly available.

Conclusions

We found that host-response-based diagnostics could accurately identify patients with ATB and predict individuals with high risk of progression from LTBI to ATB prior to sputum conversion. We found that a higher number of genes in a signature did not increase the accuracy of the signature. Overall, the Sweeney3 signature performed robustly across all comparisons. Our results provide strong evidence for the potential of host-response-based diagnostics in achieving the WHO goal of ending tuberculosis by 2035, and host-response-based diagnostics should be pursued for clinical implementation.

Author summary

Why was this study done?

- There is an urgent need for a non-sputum-based triage test for diagnosis of active tuberculosis (ATB).
- The World Health Organization (WHO) has specified criteria for such a test that could be used to end tuberculosis by 2035.
- Several gene signatures measuring host immune response to *Mycobacterium tuberculosis* in blood samples have been proposed, but none has translated in clinical practice.

What did the researchers do and find?

- Researchers compared 16 such gene signatures to investigate whether 1 or more of them could identify patients with ATB with the desired accuracy.
- The analysis found that 2 of the proposed gene signatures satisfied the WHO criteria for a non-sputum-based triage test across heterogeneous culture-confirmed datasets.
- Importantly, only 1 of these 2 signatures had a low false positive rate and no heterogeneity in its diagnostic accuracy, suggesting that it is generalizable across diverse patient populations.

What do these findings mean?

- The findings strongly suggest that host-response-based diagnostics for ATB have the potential to aid in achieving the WHO goal of ending TB by 2035 and should be considered for clinical implementation.

Introduction

The World Health Organization (WHO) has identified the need for a non-sputum-based triage test to rule out active tuberculosis (ATB) disease [1]. The WHO consensus meeting report describes that such a triage test should have 90% sensitivity and 70% specificity at minimum to end tuberculosis (TB) by 2035 [1]. In clinical practice, a triage test to rule out ATB requires high negative predictive value (NPV). WHO has also described the need for a test to predict progression from latent TB infection (LTBI) to ATB with >75% specificity and >75% sensitivity [2]. Further, the Foundation for Innovative New Diagnostics (FIND) and the New Diagnostics Working Group of the Stop TB Partnership have proposed a need for a prognostic test for TB risk that requires a positive predictive value (PPV) > 5.8% at a 2-year cumulative incidence of ATB of 2% (http://www.finddx.org/wp-content/uploads/2016/05/TPP-LTBIprogression.pdf).

Sputum culture is considered the gold standard for ATB diagnosis but takes 6–7 days for a positive diagnosis and up to 42 days for a confirmed negative diagnosis. Current sputum-based tests in clinical practice (e.g., smear microscopy, culture, and PCR-based assays) do not meet the desired target product profiles (TPPs), lack the sensitivity to reliably distinguish ATB from LTBI, and are prone to producing false negative results because sufficient bacilli-containing sputum samples can be difficult to obtain, especially from children and from individuals co-infected with HIV [3–12]. Sputum-bacilli tests cannot be used to identify patients with a high risk of progression because diagnosis with ATB is defined by the presence of bacilli in sputum [12]. A stool-based diagnostic test for ATB in children was shown to have 31.9% sensitivity at 99.7% specificity. These performance statistics are well suited for a diagnostic test for ruling in a patient with ATB, but not for a triage test to rule out ATB or for a progression test [13].

Recently, diagnostic gene signatures based on host immune response have been repeatedly demonstrated to accurately distinguish infection from other non-pathogenic inflammatory conditions [14], and to distinguish bacterial and viral infections [15,16]. Particularly for ATB, several host-response-based gene signatures have been proposed over the last decade for distinguishing patients with ATB from healthy controls and patients with LTBI and other diseases (ODs), and to predict progression from LTBI to ATB [17–29]. Collectively, these studies profiled whole blood or peripheral blood mononuclear cells (PBMCs) from samples that span a broad range of clinical conditions, including different age groups (children, adolescents, and adults), infection types (LTBI and ATB), and control (noninfectious) conditions. The number of genes in these signatures varies dramatically [17,20,23]. Notably, a variety of computational techniques—including support vector machine, random forest, linear discriminant analysis, logistic regression, and difference of means—have been applied to identify these signatures.

Despite extensive efforts, none of these TB gene signatures has been translated into a point of care (POC) diagnostic for several reasons. First, none of these signatures except 1 has been validated in prospective independent cohorts. As more gene signatures have been described, there has been a dearth of studies comparing these signatures with each other to verify if host-response-based signatures are appropriate for translation to clinical practice. Second, a

majority of these gene signatures are composed of a large number of genes. Although a higher number of genes in a signature tends to increase accuracy [24], implementation of such a signature as a simple and cost-effective POC test is very difficult using current technology for measuring gene expression. Virtually all commercially available platforms for measuring transcriptional host response are limited by the number of genes they can measure. For instance, the Cepheid GeneXpert system, arguably the most widely used platform in TB diagnostics, can measure only up to 10 genes. Also, 1 or 2 of these genes need to be control genes, which further reduces the number of genes that can be used in a diagnostic signature to 8 or 9. Third, and most importantly, the generalizability of these transcriptional signatures to real-world patient populations in various clinical contexts is questionable. For instance, a 16-gene signature for predicting progression from LTBI to ATB developed using a single cohort from a single country was shown to lack generalizability to cohorts from other countries on the same continent [29]. In contrast, a 3-gene signature developed using heterogeneous cohorts from multiple countries has been shown to be more generalizable to other countries in retrospective [17] and prospective validation [11,30]. Another factor with substantial impact on the generalizability of these signatures is that the different statistical models used for creating these signatures are difficult to generalize across different populations and different measurement technologies. For instance, models based on *K*-nearest-neighbors clustering are difficult to generalize due to high sensitivity to batch effects and scaling within data [31].

The proliferation of host-response-based gene signatures despite the challenges described above raises several questions. First, do these signatures perform similarly to each other in different clinical contexts in different patient populations? If yes, the second question is, do 1 or more of these signatures have the potential to move towards translation into clinical practice cost-effectively? Third, an overarching question is, does host response to *Mycobacterium tuberculosis (Mtb)* have the potential to achieve the generalizability required to be used as a non-sputum-based triage test that meets the TPPs described by the WHO and other groups for ending TB by 2035?

We sought to answer these questions through a systematic comprehensive analysis of gene signatures for diagnosis of TB. To estimate the diagnostic accuracy of each of the published signatures, we reconstructed the classification model associated with each gene signature using the same discovery cohort as the original publication to the best of our ability. We then evaluated the accuracy of each signature in distinguishing patients with ATB from those with LTBI or ODs and healthy controls using publicly available gene expression datasets. We also evaluated whether these signatures could predict progression from LTBI to ATB prior to sputum conversion. We used specificity, sensitivity, comparison with various TPPs for ending TB by 2035, weighted mean area under the receiver operating characteristic curve (AUROC), PPV, NPV, false positive rate (FPR), heterogeneity across datasets, and diagnostic odds ratio (DOR) to evaluate the accuracy of various gene signatures.

Methods

Prospective analysis plan

We did not have a prospective analysis plan. Our goal from the beginning was broadly divided into the following steps that were modeled after a similar benchmarking analysis of biomarkers for sepsis [32]:

Step 1: Identify a set of gene signatures for diagnosis of ATB from literature. We define a "gene signature" as a set of genes derived from an analysis of whole transcriptome profiles that distinguishes patients with ATB from healthy controls or patients with LTBI or ODs.

Step 2: Identify a set of appropriate transcriptome datasets from PBMC or whole blood samples. A "transcriptome dataset" is a collection of "transcriptome profiles," such that expression of a large number of genes is measured in each sample (typically >10,000 genes).

Step 3: Implement the corresponding diagnostic model for a gene signature identified in Step 1 by following the Methods section in the paper describing the gene signature.

Step 3A: If needed, retrain a diagnostic model. We found that some signatures were missing required information in the corresponding paper for us to evaluate those signatures as "locked" models in independent cohorts. For example, when coefficients for a signature based on logistic regression or linear discriminant analysis were missing, we used the discovery cohort to learn the coefficients.

Step 3B: If needed, identify a comparable dataset for retraining. This step was required for a subset of signatures because the data on which they were originally trained were not available.

Step 4: "Lock" each diagnostic model and apply it to the transcriptome datasets identified in Step 2.

Step 5: Aggregate AUROC, specificity at 90% sensitivity, and NPV for each signature across independent datasets, while excluding the corresponding discovery datasets for each signature.

Step 5A: Aggregate AUROC, specificity at 90% sensitivity, and NPV using only datasets where diagnosis of ATB is culture-confirmed.

Step 5B: Aggregate AUROC, specificity at 90% sensitivity, and NPV using all datasets irrespective of how ATB is diagnosed (culture-confirmed or clinical diagnosis).

Step 6: Identify gene signatures that meet the WHO TPP of 70% specificity at 90% sensitivity for a non-sputum-based triage test.

Step 7: Aggregate PPV and NPV at 2% prevalence for each signature across independent datasets, while excluding the corresponding discovery datasets for each signature.

Step 7A: Aggregate PPV and NPV using only datasets where diagnosis of ATB is culture-confirmed.

Step 7B: Aggregate PPV and NPV using all datasets irrespective of how ATB is diagnosed (culture-confirmed or clinical diagnosis).

Step 8: Identify gene signatures that meet the FIND TPP of 5.8% PPV at 2% prevalence for a test for predicting progression from LTBI to ATB.

Step 9: Identify gene signatures that meet the WHO TPP of >75% specificity and >75% sensitivity for a test for predicting progression from LTBI to ATB.

In response to the comments by the reviewers, we performed bivariate meta-analysis to assess DOR and its heterogeneity for each gene signature, along with FPR.

Gene signatures for comparison

In June 2018 we performed an extensive search of published gene signatures for distinguishing patients with ATB from healthy controls or patients with LTBI or ODs. We searched the National Center for Biotechnology Information (NCBI) repository of publications (PubMed) for all publications describing a gene signature for the diagnosis of ATB. We included all blood-based gene signatures that were specifically designed to diagnose ATB. We did not exclude any studies because of study criteria or date. Search terms included the following: TB (tuberculosis) gene signature, TB (tuberculosis) transcriptional signature, and TB (tuberculosis) diagnostic. We identified 11 publications describing 16 gene signatures for the diagnosis of ATB (Table 1) [17–27]. We note that we only considered gene signatures that were described for diagnosis of ATB compared to healthy controls or patients with LTBI or ODs. For instance, we did not include the 4-gene RISK4 signature by Suliman et al. [29] or the 16-gene correlates

Table 1. Gene expression signatures compared within this study.

Citation	PubMed PMID	GEO discovery dataset	Signature name	Indication	Number of genes	Statistical model	Retraining required
Anderson et al. [19]	24785206	GSE39940	Anderson42	ATB vs LTBI	42	Difference of sums	No
			Anderson51	ATB vs ODs	51	Difference of sums	No
Berry et al. [20]	20725040	GSE19491	Berry393	ATB vs (LTBI & HCs)	393	*K*-nearest neighbors	Yes
			Berry86	ATB vs ODs	86	*K*-nearest neighbors	Yes
Bloom et al. [21]	23940611	GSE42834	Bloom144	ATB vs (ODs & HCs)	144	Support vector machine	Yes
Laux da Costa et al. [22]	26025597	GSE42834*	daCosta3	ATB vs ODs	3	Random forest	Yes
Jacobsen et al. [23]	17318616	GSE6112*	Jacobsen3	ATB vs LTBI	3	Linear discriminant analysis	Yes
Kaforou et al. [18]	24167453	GSE37250	Kaforou27	ATB vs LTBI	27	Difference of means	No
			Kaforou44	ATB vs ODs	44	Difference of means	No
			Kaforou52	ATB vs (LTBI & ODs)	52	Difference of means	No
Leong et al. [24]	29559120	GSE101705	Leong24	ATB vs LTBI	24	Rigid logistic regression	Yes
Maertzdorf et al. [25]	26682570	GSE74092	Maertzdorf15	ATB vs (LTBI & HCs)	15	Random forest	Yes
			Maertzdorf4	ATB vs (LTBI & HCs)	4	Random forest	Yes
Sambarey et al. [26]	28065665	GSE37250*	Sambarey10	ATB vs (LTBI & HCs & ODs)	10	Linear discriminant analysis	Yes
Sweeney et al. [17]	26907218	GSE19491, GSE37250, GSE42834	Sweeney3	ATB vs (LTBI & ODs & HCs)	3	Difference of geometric means	No
Verhagen et al. [27]	23375113	GSE41055	Verhagen10	ATB vs (LTBI & HCs)	10	Random forest	Yes

Gene signatures are named by combining the last name of the first author followed by the number of genes in the signature.

*The diagnostic model for the signature was created using this dataset as the original training dataset was not available.

ATB, active tuberculosis; GEO, Gene Expression Omnibus; HC, healthy control; LTBI, latent tuberculosis infection; OD, other disease.

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of risk signature by Zak et al. [28] as both signatures are designed to predict progression from LTBI to ATB, not to diagnose ATB as a triage test.

Recreating corresponding classification models for each gene signature

For each of the 16 published signatures, we constructed a classification model as described in the original paper to the best of our ability. We created and trained each classification model to be as accurate a replica of the model in the original publication as possible, using the same datasets used in the original publication except where we were unable to access the original training data (Table 1). In those instances, we trained the model on a different, suitable dataset as indicated in Table 1. We confirmed that the classification models were successfully reconstructed by comparing the performance of each model to the performance of the model as described in the original publication (S1 Table). S1 Text provides a detailed description of each model.

Transcriptome datasets used for comparing signatures

We searched NCBI GEO and European Bioinformatics Institute (EBI) ArrayExpress in June 2018 using the following search terms: TB (tuberculosis) gene expression, TB (tuberculosis) microarray, TB (tuberculosis) blood microarray, TB (tuberculosis) RNAseq, TB (tuberculosis) blood RNAseq, TB (tuberculosis) peripheral blood mononuclear cells gene expression, TB (tuberculosis) peripheral blood mononuclear cells microarray, and TB (tuberculosis) peripheral blood mononuclear cells RNAseq. We included all datasets that measured transcriptomes from the blood of patients with ATB and at least 1 other group of individuals. We did not

exclude datasets based on collection date or sample number. We excluded datasets profiled using quantitative PCR because they did not have enough coverage to capture all genes across the 16 signatures included in our analysis. We identified 24 datasets containing 3,083 transcriptome profiles from whole blood or PBMCs of patients with ATB and healthy controls or patients with LTBI or ODs from 14 countries through an extensive search of 2 public data repositories (NCBI GEO and EBI ArrayExpress) (Table 2). These datasets also include the 8 datasets that were used to derive the 16 gene signatures. We used these 24 datasets to compare each of the 16 gene signatures for their ability to distinguish ATB from all other groups (healthy controls, LTBI, and ODs). In most instances the method of diagnosis of patients with ATB could be confirmed (as indicated under "diagnosis method" in Table 2); in some cases the method of diagnosis could not be identified from the information available publicly. The datasets included in this analysis were collected in 14 different countries and measures on 18 different platforms. In total these datasets include 3,083 individuals, of whom 944 were patients with ATB at the time of sample collection.

Evaluation of model performance

We assessed the performance of each signature in each dataset using AUROC statistics that were calculated for each model across all datasets not used in the discovery or training of the model, as well as across only culture-confirmed cases not used in the discovery or training of the model. In an effort to avoid bias against any model, the AUROC was calculated at the optimal cut-point for each dataset/model combination. We used the R package OptimalCutpoints to determine the AUROC and Youden index for each signature in each dataset. The optimal cut-point was identified using the Youden method [33]. A weighted mean AUROC was then calculated for each model. No universal cutoffs were used, but rather the most favorable cutoff for each model under each condition.

Weighted mean statistics

The weighted mean AUROC was calculated as shown below in Eq 1, where d is the number of datasets analyzed, AUROC_i is the AUROC for a given dataset i, and n_i is the number of samples within dataset i [34]:

$$\text{weighted mean AUROC} = \frac{\sum_{i=1}^{d} \mathrm{AUROC}_i * n_i}{\sum_{i=1}^{d} n_i} \quad (1)$$

The 95% confidence interval for the weighted mean AUROC was calculated by adding and subtracting 1.96 times the standard deviation (SD) of the mean from the weighted mean AUROC according to Eqs 2 and 3 below, where ω refers to the weighted sum of observations squared, σ refers to the sum of observations squared, and δ refers to the degrees of freedom:

$$\mathrm{SD} = \sqrt[2]{\frac{(\omega - \sigma)}{(\delta)}} \quad (2)$$

$$95\%\ \mathrm{CI} = \text{ weighted mean AUROC} \pm (1.96 * \mathrm{SD}) \quad (3)$$

Table 2. Transcriptome datasets used for comparison of 16 gene signatures for diagnosis of ATB.

GEO accession	GEO platform	Country	Tissue	Age (years)	HIV status	Diagnosis method	HCs	LTBI	ATB	ODs	Total	Notes
GSE19491	GPL6947	UK, South Africa	Whole blood	>17	Negative	Sputum culture	117	69	61	193	440	ODs included staph, strep, Still disease, systemic lupus erythematosus, and pediatric systemic lupus erythematosus
GSE28623	GPL4133	The Gambia	Whole blood	16–53	Negative	Sputum microscopy, chest X-ray	37	25	46		108	
GSE29536	GPL6102	UK	Whole blood	11–88	Negative		6		9		15	Only the TB dataset within this series was used
GSE34608	GPL6480	Germany	Whole blood	17–73	Negative		18		8	18	108	OD samples were sarcoidosis samples; controls may have included some IGRA-positive individuals
GSE37250	GPL10558	Malawi	Whole blood	>17	Some positive	Sputum culture		167	195	175	537	
GSE39939	GPL10558	Kenya	Whole blood	<15	Some positive	Sputum culture		14	79	64	157	
GSE39940	GPL10558	Malawi	Whole blood	<15	Some positive	Sputum culture		54	111	169	334	
GSE41055	GPL5175	Venezuela	Whole blood	<15	Negative		9	9	9		27	
GSE42834	GPL10558	Germany	Whole blood	>17	Negative	Sputum culture	118		40	123	281	ODs included sarcoidosis, pneumonia, and lung cancer
GSE50834	GPL10558	South Africa	PBMCs	30–40	Positive			23	21		44	
GSE56153	GPL6883	Indonesia	Whole blood	>15	Negative	Sputum microscopy, chest X-ray, clinical presentation	18		18		36	HIV status not measured in controls, but HIV has very low prevalence in Indonesia
GSE54992	GPL570	China	PBMCs	18–68	Negative	Sputum microscopy, chest X-ray, clinical presentation, sputum culture	6	6	9		21	Dataset also included 18 treated samples not used in this analysis; samples confirmed with sputum culture were not identified
GSE62147	GPL6480	Germany	Whole blood	15–79	Negative	Sputum culture			14	12	26	OD was *Mycobacterium africanum*
GSE62525	GPL16951	Taiwan	PBMCs		Negative		14	14	14		42	
GSE69581	GPL10558	South Africa	Whole blood	>17	Positive	Microbiologically confirmed (method not specified)		25	15		40	Dataset also included samples with subclinical TB
GSE73408	GPL11532	US	Whole blood	>17	Negative	Sputum culture	39	35	35		109	
GSE79362	GPL11154	South Africa	Whole blood	12–18	Negative	Sputum smear, sputum culture		101	19		120	Longitudinal samples were collected
GSE81746	GPL17077	India	Whole blood	25–65	Unknown		2		4		6	
GSE83456	GPL10558	UK	Whole blood		Negative	Granuloma biopsy, clinical presentation with response to therapy, radiology with response to treatment, sputum culture	61		45	49	155	ODs included sarcoidosis; there were also extrapulmonary TB samples available from this dataset.

(*Continued*)

Table 2. (Continued)

GEO accession	GEO platform	Country	Tissue	Age (years)	HIV status	Diagnosis method	HCs	LTBI	ATB	ODs	Total	Notes
GSE83892	GPL10559	UK	Whole blood	>17	Positive	Cerebral spinal fluid smear, cerebral spinal fluid culture, conventional and real-time PCR of cerebral spinal fluid		17	99		116	ATB patients in this cohort included patients with complications from immune reconstitution inflammatory syndrome and TB meningitis
GSE84076	GPL16791	Brazil	Whole blood	>18	Negative	Sputum microscopy, clinical presentation, sputum culture	6	6	9		21	This dataset also included some samples taken from individuals after treatment
GSE101705	GPL18573	India	Whole blood	>6	Negative	Sputum culture		16	28		44	
GSE107731	GPL15207	Mongolia	Whole blood		Unknown		3		3		6	
GSE107994	GPL20301	UK	Whole blood	16–84	Negative	Sputum culture, sputum PCR	119	118	53		290	Dataset included samples from latent TB progressors
24 Datasets		**14 countries**					**573**	**699**	**944**	**803**	**3,083**	

ATB, active tuberculosis; GEO, Gene Expression Omnibus; HC, healthy control; IGRA, Interferon Gamma Release Assay; LTBI, latent tuberculosis infection; OD, other disease; PBMC, peripheral blood mononuclear cell; TB, tuberculosis.

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Results

Our systematic search of the literature for published transcriptional signatures diagnosing ATB against other clinical conditions identified 16 transcriptional signatures (Table 1) that distinguished patients with ATB from 1 or more of the following: healthy controls, patients with LTBI, or patients with ODs. Next, we searched 2 public data repositories (NCBI GEO and EBI ArrayExpress) for gene expression datasets that profiled whole blood or PBMC samples comparing patients with ATB to healthy controls or patients with LTBI or ODs. We identified 24 independent datasets consisting of 3,083 transcriptome profiles from 14 countries (Table 2). Note that 8 of these 24 datasets were used to derive 1 or more of the 16 gene signatures. Therefore, in order to ensure that the discovery cohort(s) of each signature did not bias its overall performance, we removed the corresponding discovery cohort(s) for each signature when computing the overall performance of each signature across all datasets. For example, for the Verhagen10 signature, we removed GSE41055 when estimating the overall AUROC and PPV, whereas for Sweeney3 we removed GSE19491, GSE37250, and GSE42834.

Overall, 630 genes were described across the 16 signatures, with the number of genes in a signature ranging from 3 [17,22,23] to 393 [20] (Fig 1). A majority of the genes (81%) were in only 1 signature. Every gene signature partially overlapped with at least 1 other signature. One gene signature (Maertzdorf4) did not include a unique gene as it was derived from another gene signature (Maertzdorf15).

Comparison of accuracy across only datasets with culture-confirmed diagnosis of ATB

For each of the 16 gene signatures, we built a classification model by following the methods described in the corresponding paper to the best of our ability. We found that the classification model we created for each signature closely reproduced the AUROC reported in the corresponding paper, though not exactly (S1 Table). We applied these "locked" models "as is" to

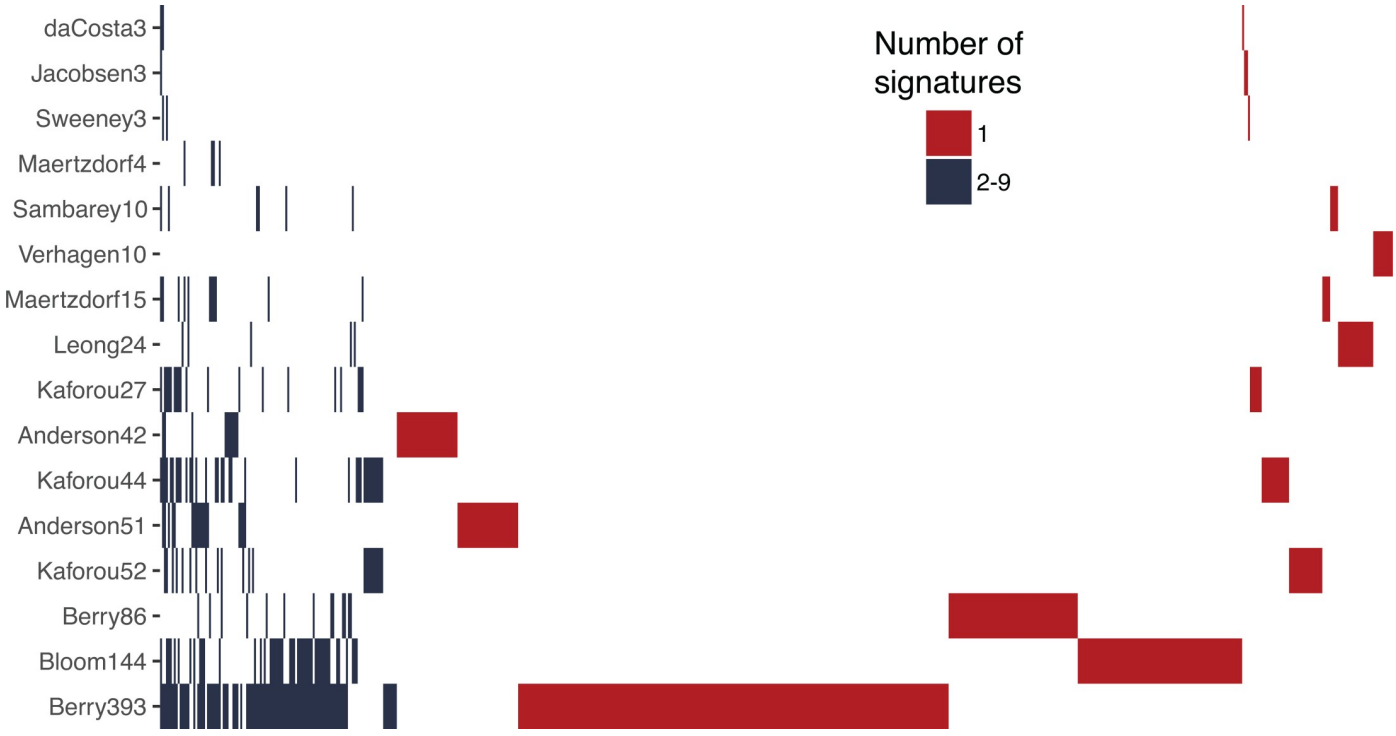

Fig 1. Distribution of genes across the signatures included in this study. Each row represents a gene signature for active tuberculosis diagnosis. Each column represents 1 gene. The number at the end of a signature name represents the number of genes in the given signature. Genes present in only 1 signature are red; those in 2 or more signatures are blue.

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other datasets to assess the generalizability of each model for accurately distinguishing patients with culture-confirmed ATB across independent datasets.

Across the 9 independent datasets with culture-confirmed ATB, 11 gene signatures had weighted mean AUROC > 0.8, which suggests that many of the host-response-based signatures tended to have generalizability (Table 3). However, 2 gene signatures (Verhagen10 and Anderson51) had weighted mean AUROC ≤ 0.6. All but 2 gene signatures (Berry86 and Berry393) had high NPV (>98% at 2% prevalence) in culture-confirmed datasets. Signatures with low AUROC or NPV (Verhagen10, Leong24, Anderson51, Berry86, and Berry393) suggest that several signatures did not generalize to independent cohorts with culture-confirmed diagnosis of ATB. Arguably, the lack of generalizability in these signatures may be expected for several reasons. First, some signatures were derived using samples from children (Verhagen10 and Anderson51). Second, a signature may not have been derived to be generalizable. For instance, Anderson et al. [19] described 2 gene signatures (Anderson42 and Anderson51) from the same dataset of children under 5 years of age for distinguishing patients with ATB from patients with LTBI (Anderson42) or with ODs (Anderson51), which may not generalize to adults. In contrast, the Sweeney3 signature, which was derived using 3 independent cohorts of adults, generalized to children (age ≤ 5 years) and adolescents (age 12–18 years). Across all culture-confirmed datasets, the Sweeney3 and Sambarey10 signatures had the highest accuracy in distinguishing patients with ATB from healthy controls and patients with LTBI or ODs (specificity 74% at 90% sensitivity). Both signatures were the only signatures to meet the minimal WHO TPP for a triage test in cohorts of patients with culture-confirmed diagnosis of ATB.

Table 3. Weighted mean AUROC, specificity at 90% sensitivity, and NPV at 2% prevalence for ATB versus all other conditions across all datasets and across only culture-confirmed datasets for each of the 16 gene signatures.

Signature	Culture-confirmed datasets			All datasets		
	AUROC (95% CI)	Specificity (95% CI)	NPV	AUROC (95% CI)	Specificity (95% CI)	NPV
Sweeney3	0.89 (0.82–0.96)	0.74 (0.40–0.89)	0.99	0.85 (0.72–0.99)	0.66 (0.23–0.93)	0.98
Jacobsen3	0.86 (0.72–1.00)	0.68 (0.37–0.93)	0.99	0.83 (0.69–0.98)	0.59 (0.21–0.92)	0.99
daCosta3	0.83 (0.60–1.00)	0.65 (0.31–0.88)	0.99	0.76 (0.45–1.00)	0.50 (0.00–0.95)	0.94
Maertzdorf4	0.83 (0.74–0.91)	0.58 (0.28–0.82)	0.99	0.79 (0.64–0.95)	0.54 (0.24–0.79)	0.99
Sambarey10	0.90 (0.83–0.97)	0.74 (0.36–0.94)	0.99	0.82 (0.57–1.00)	0.59 (0.18–0.94)	0.99
Verhagen10	0.53 (0.46–0.60)	0.13 (0.11–0.19)	0.98	0.54 (0.41–0.68)	0.14 (0.00–0.32)	0.92
Maertzdorf15	0.82 (0.71–0.92)	0.58 (0.30–0.82)	0.99	0.79 (0.66–0.92)	0.54 (0.23–0.83)	0.99
Leong24	0.74 (0.53–0.95)	0.41 (0.12–0.63)	0.99	0.75 (0.54–0.95)	0.43 (0.04–0.77)	0.99
Kaforou27	0.86 (0.71–0.92)	0.66 (0.40–0.92)	0.99	0.83 (0.64–1.00)	0.62 (0.28–0.94)	0.99
Anderson42	0.84 (0.75–0.93)	0.61 (0.39–0.82)	0.99	0.82 (0.66–0.97)	0.58 (0.27–0.87)	1.00
Kaforou44	0.82 (0.67–0.97)	0.61 (0.27–0.80)	0.99	0.78 (0.56–1.00)	0.54 (0.12–0.85)	0.99
Anderson51	0.60 (0.42–0.79)	0.22 (0.00–0.44)	0.99	0.58 (0.33–0.82)	0.21 (0.00–0.52)	0.96
Kaforou52	0.87 (0.77–0.97)	0.67 (0.45–0.87)	0.99	0.84 (0.70–0.99)	0.62 (0.29–0.92)	0.99
Berry86	0.67 (0.44–0.90)	0.21 (0.00–0.76)	0.47	0.69 (0.36–1.00)	0.29 (0.00–0.65)	0.47
Bloom144	0.81 (0.61–1.00)	0.50 (0.10–0.64)	0.99	0.74 (0.52–0.96)	0.33 (0.00–0.65)	0.98
Berry393	0.72 (0.43–1.00)	0.40 (0.00–1.00)	0.74	0.71 (0.43–0.99)	0.34 (0.00–0.98)	0.66

ATB, active tuberculosis; AUROC, area under the receiver operating characteristic curve; NPV, negative predictive value.

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Comparison of accuracy across all datasets for diagnosis of ATB

Next, we compared accuracy of the 16 signatures across all datasets irrespective of how ATB was diagnosed, which included sputum microscopy and clinical presentation (Table 3). Weighted mean AUROC for all but 3 signatures was lower than when only considering culture-confirmed datasets, although these decreases were not meaningful ($\leq$4% decrease). Importantly, 3 signatures (Sambarey10, daCosta3, and Bloom144) had a substantial reduction in AUROC (7%, 8%, and 7%, respectively), which results in their specificity at 90% sensitivity decreasing by greater than 15%. None of the gene signatures met the minimal WHO TPP for the triage test when including all datasets. The AUROC for the Sweeney3 gene signature decreased by 4%, resulting in an overall specificity of 66% at 90% sensitivity, which was the highest among all signatures when datasets were included irrespective of how ATB was diagnosed. Another signature (Kaforou52) also had specificity 62% at 90% sensitivity, but included 52 genes, a substantially higher number of genes than the Sweeney3 signature. Notably, we found no correlation between the number of genes in a signature and the weighted mean AUROC ($R = -0.04$, $p = 0.86$). Finally, 5 gene signatures (daCosta3, Verhagen10, Anderson51, Berry86, and Berry393) had NPV (<98%) too low to be clinically useful as a triage test.

Comparison of accuracy for diagnosis of ATB using bivariate meta-analysis

The comparison of the 16 signatures can also be performed as a bivariate meta-analysis by combining sensitivity and specificity from diagnostic tests across different datasets. We used the R package mada to compare the 16 signatures by computing their DOR, heterogeneity in DOR, and overall FPR. A clinically useful generalizable triage test should have low FPR and high DOR with no heterogeneity across characteristics of patient populations such as genetic background of host, *Mtb* strain, age, HIV co-infection, and bacillus Calmette–Guérin vaccination.

Four signatures (Sweeney3, Kaforou52, Kaforou44, and Anderson51) had no heterogeneity irrespective of what datasets were used for analysis (only culture-confirmed or all datasets); the remaining 12 signatures showed heterogeneity (Table 4). Among the 4 signatures with no heterogeneity, the Sweeney3 signature had the highest DOR with the lowest FPR (Table 4) irrespective of what datasets were used, and was the only signature using fewer than 10 genes. Interestingly, when only culture-confirmed datasets were used, the daCosta3 signature had the highest DOR (32.44), with no heterogeneity and a 26% FPR. However, when datasets with clinical diagnoses of ATB were included, the daCosta3 signature had increased heterogeneity (13.63%) and a very high FPR (45%). On the other hand, the Maertzdorf15 signature had substantial heterogeneity (19.07%) when using only culture-confirmed datasets, which decreased to no heterogeneity when datasets with clinical diagnoses were included, without substantial changes in DOR or FPR. These changes in heterogeneity and FPR depending on which datasets are used in the analysis further suggest that certain signatures may not be generalizable to broad patient populations.

Signature performance predicting progression 6 months prior to ATB diagnosis

Predicting progression from LTBI to ATB prior to sputum conversion is an important step in reducing overall incidence of ATB. Previously, a 16-gene (CoR [28]) and a 4-gene (RISK4 [29]) signature have been described to identify individuals with a high likelihood of progression. CoR was derived from and validated in the Adolescent Cohort Study (ACS; GSE79362) with validation AUROC = 69% with 66% sensitivity and 81% specificity. However, this signature was shown to have poor generalizability in the GC6-74 cohort from other African countries in a follow-up study. Therefore, we excluded CoR from our comparison. The second signature, RISK4, was derived from the GC6-74 cohort and was shown to have AUROC = 69% in the ACS [29]. Using the ACS, we compared the 16 gene signatures for their ability to predict

Table 4. Comparison of 16 gene signatures for diagnosis of ATB using bivariate meta-analysis.

Signature	Culture-confirmed datasets			All datasets		
	DOR (95% CI)	Heterogeneity	FPR (95% CI)	DOR (95% CI)	Heterogeneity	FPR (95% CI)
Sweeney3	30.50 (14.95–62.24)	0	0.18 (0.13–0.26)	16.66 (11.56–24.00)	0	0.20 (0.16–0.24)
Kaforou52	21.05 (12.20–36.34)	0	0.23 (0.16–0.33)	14.05 (10.10–19.54)	0	0.23 (0.18–0.28)
Kaforou44	12.22 (6.04–24.71)	0	0.22 (0.16–0.30)	9.05 (6.42–12.74)	0	0.22 (0.17–0.29)
Anderson51	4.96 (2.86–8.59)	0	0.26 (0.09–0.53)	3.91 (2.90–5.26)	0	0.32 (0.20–0.46)
Jacobsen3	19.89 (10.72–36.89)	4.03	0.22 (0.16–0.30)	13.04 (9.54–17.82)	0	0.21 (0.17–0.26)
Kaforou27	17.21 (11.08–26.74)	1.13	0.25 (0.17–0.34)	13.85 (10.32–18.59)	0	0.23 (0.18–0.29)
Maertzdorf15	14.38 (8.04–25.70)	19.07	0.27 (0.21–0.34)	11.65 (8.37–16.22)	0	0.26 (0.20–0.31)
Maertzdorf4	13.82 (8.75–21.83)	0.73	0.24 (0.18–0.31)	9.69 (7.39–12.71)	3.06	0.28 (0.23–0.33)
Anderson42	11.26 (7.50–16.92)	4.8	0.28 (0.24–0.33)	10.65 (7.87–14.42)	8.39	0.26 (0.21–0.31)
Sambarey10	19.13 (10.38–35.25)	16.87	0.19 (0.13–0.28)	12.18 (8.54–17.37)	11.61	0.20 (0.17–0.24)
daCosta3	32.44 (14.90–70.63)	0	0.26 (0.13–0.44)	13.89 (8.14–23.71)	13.63	0.45 (0.28–0.64)
Verhagen10	1.85 (1.30–2.63)	21.43	0.47 (0.28–0.67)	2.90 (2.03–4.15)	21.02	0.47 (0.31–0.65)
Bloom144	9.94 (5.49–17.99)	50.55	0.21 (0.13–0.32)	6.69 (4.71–9.49)	23.29	0.24 (0.16–0.34)
Leong24	8.20 (4.75–14.16)	46.33	0.27 (0.18–0.39)	8.48 (5.96–12.06)	23.93	0.26 (0.19–0.34)
Berry393	17.72 (7.41–42.35)	33.39	0.16 (0.09–0.27)	9.26 (5.90–14.53)	25.48	0.45 (0.25–0.66)
Berry86	12.62 (4.98–31.99)	42.89	0.19 (0.04–0.57)	6.72 (3.81–11.85)	27.48	0.66 (0.35–0.87)

ATB, active tuberculosis; DOR, diagnostic odds ratio; FPR, false positive rate.

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progression from LTBI to ATB 6 months prior to sputum conversion. Because CoR was derived from the ACS cohort and shown to be not generalizable to cohorts from other African countries, we excluded the CoR signature from the comparison. We also excluded the RISK4 signature from further comparison as its performance characteristics are previously described in the ACS cohort [29].

Seven out of 16 signatures had AUROC > 0.8 and PPV > 5.8% at 2% prevalence in the ACS cohort (Table 5). Higher numbers of genes in a signature again did not correspond to a substantial increase in the AUROC. Only 2 of these signatures (Sweeney3 and Jacobsen3) had fewer than 10 genes. Interestingly, although the daCosta3 signature had the highest PPV (14.60%) for predicting progression from LTBI to ATB, it had AUROC = 0.56. Sweeney3 had the lowest number of genes with the highest AUROC (0.86, 95% CI 0.78–0.93) and PPV (13.6%), which exceeded the FIND TPP for the progression test. Overall, these results demonstrated that, in patients with LTBI, host response is able to identify those at high risk of progression to ATB prior to sputum conversion.

Discussion

In this study, we compared 16 gene signatures for distinguishing patients with ATB from healthy controls or patients with LTBI or ODs using 24 independent datasets of >3,000 whole blood or PBMC transcriptome profiles from 14 countries. Collectively, these datasets represented real-world heterogeneity observed in patients with TB. For instance, the samples collected across 14 countries represented diversity in both host and pathogen genetics. Similarly, some datasets profiled samples from children whereas others profiled samples from adults, which represented heterogeneity in host response due to age. These data also represented heterogeneity in clinical practice as patients were diagnosed using different criteria (e.g., sputum culture versus sputum microscopy).

Across these biologically and technologically heterogeneous data, our comparison found that several gene signatures distinguished patients with ATB with moderate to high accuracy,

Table 5. AUROC, PPV, and NPV for progression from LTBI to ATB in the ACS cohort up to 180 days prior to diagnosis.

Signature	AUROC (95% CI)	PPV at 2% prevalence	NPV at 2% prevalence
daCosta3	0.56 (0.50–0.62)	14.60	98.2
Sweeney3	0.86 (0.78–0.94)	13.60	99.4
Kaforou27	0.86 (0.78–0.94)	13.60	99.4
Kaforou52	0.87 (0.80–0.95)	13.20	100
Leong24	0.73 (0.62–0.83)	11.00	99.3
Jacobsen3	0.85 (0.76–0.93)	10.80	NC
Anderson42	0.85 (0.77–0.92)	8.80	99.5
Bloom144	0.68 (0.56–0.79)	8.30	98.9
Sambarey10	0.80 (0.72–0.89)	6.40	99.4
Kaforou44	0.83 (0.76–0.90)	6.20	99.4
Maertzdorf15	0.63 (0.56–0.69)	2.80	99.5
Anderson51	0.46 (0.34–0.58)	2.70	98.2
Maertzdorf4	0.51 (0.50–0.52)	2.00	99.5
Verhagen10	0.47 (0.45–0.49)	2.00	NC
Berry86	0.50 (0.50–0.50)	2.00	98.9
Berry393	0.50 (0.50–0.50)	2.00	NC

ACS, Adolescent Cohort Study; ATB, active tuberculosis; AUROC, area under the receiver operating characteristic curve; LTBI, latent tuberculosis infection; NC, not calculated; NPV, negative predictive value; PPV, positive predictive value.

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although almost all signatures included a large number of genes, which severely restricts their ability for cost-effective translation to clinical practice at the POC. Importantly, our analysis found that a higher number of genes in a signature did not translate into higher accuracy across biologically heterogeneous data. Only 2 gene signatures (Sweeney3 and Sambarey10) satisfied the WHO TPP for a non-sputum-based triage test to identify which patients need further testing for confirming ATB, when comparing the signatures using only datasets from patients with culture-confirmed diagnosis of ATB.

When we included additional datasets that diagnosed ATB using other means (e.g., sputum microscopy or clinical diagnosis), the accuracy of 13 signatures decreased such that no signature satisfied the WHO TPP for a non-sputum-based triage test. For the 2 signatures that satisfied the WHO TPP for a non-sputum-based triage test when using only culture-confirmed datasets, the reduction in the AUROC and specificity of Sweeney3 was minimal (AUROC = 0.85, specificity = 66%, and sensitivity = 90%), whereas Sambarey10 had a substantial reduction of 15% in specificity. It is possible that inclusion of patients with ATB that was not diagnosed using positive culture caused the reduction in accuracy and may underestimate the accuracy of these signatures.

The inclusion of children in our analyses could have decreased the overall performance regarding misclassification of cases because of the challenges in diagnosis of ATB in children. GSE39939 and GSE39940, both of which were part of the same study [19], contained 157 and 334 samples (491 samples total), respectively, suggesting that the number of children in these datasets was sufficient. Out of the 491 samples, 190 samples were from children with ATB, of which 44 patients with ATB were sputum-negative; the remaining 146 children with ATB were sputum-positive. When using children with sputum-positive ATB, we did not see a substantial decrease in overall performance compared to adults. However, when children with clinically diagnosed or culture-negative ATB were included in our analysis, every gene signature had lower accuracy, similar to when including adults with clinically diagnosed or culture-negative ATB. These results suggest that gene signatures for diagnosis of ATB based on host response are not substantially affected by age, but by the possible inaccuracy of culture-negative clinical diagnosis of ATB.

We found that 7 signatures (Sweeney3, Kaforou27, Kaforou52, Jacobsen3, Anderson42, Sambarey10, and Kaforou44) identified adolescents with LTBI who progressed to ATB up to 6 months prior to sputum conversion. Among these signatures, only Sweeney3 has been prospectively validated in an active screening cohort to further demonstrate that host response to *Mtb* is detectable in blood samples earlier [13]. These results further suggest that host-response-based gene signatures could have substantial impact on the diagnosis of incipient TB, which is defined as an asymptomatic phase with early disease [35]. Incipient TB may last for up to 1 year approximately, during which a patient may be intermittently infectious by shedding bacilli in the sputum. Although the exact definitions of LTBI and incipient TB may be different, patients with LTBI progressing towards ATB in principle are similar to patients with incipient TB. Our results suggest that host-response-based gene signatures should be further explored for diagnosis of incipient TB in larger cohorts.

The ability of gene signatures for diagnosis of ATB to predict progression from LTBI to ATB prior to sputum conversion and to diagnose ATB in active screening is additional indirect evidence that suggests our estimates of accuracy for each gene signature may be underestimates. Many of the samples labeled as LTBI but classified as ATB may be progressors with subclinical ATB. Collectively, these results highlight the need for assessing the host-response-based TB diagnostics in larger prospective cohorts.

An important contributing factor to the lower generalizability of several signatures is likely the choice of underlying classification model. For instance, signatures using *K*-nearest-

neighbors clustering as a model had the overall worst performance because of the lack of co-normalization of data across datasets and platforms. Models based on *K*-nearest-neighbors clustering benefit from co-normalized data; however, it is impractical, and may be very difficult, if not nearly impossible, to co-normalize data from different clinics using different technologies.

Overall, when considering the feasibility of translating a gene signature as a POC test (e.g., number of genes, required specificity at 90% sensitivity, and robustness across datasets from different geographic regions and clinical contexts), the Sweeney3 signature consistently ranked among the best signatures. The signature has also been prospectively validated in at least 2 independent cohorts using reverse transcription PCR [11,30]. Collectively, the Sweeney3 signature has been now shown to (1) predict progression to ATB 6 months prior to sputum conversion, (2) distinguish ATB in active screen, (3) track treatment response, and (4) stratify patients with ATB at the time of diagnosis with high likelihood of subclinical ATB after treatment. This robustness of Sweeney3 may be due to the fact that it was derived using 3 independent cohorts that represented broad biological and technical heterogeneity. This is in line with the observation by Suliman et al. [29] that a gene signature derived using a homogeneous cohort from 1 country was not broadly applicable to patients from other countries with similar genetic background. It is important to note that Sweeney3 is the only prospectively validated signature among the 16 signatures compared here. Importantly, the ability of the Sweeney3 gene signature to identify patients with ATB in different clinical contexts and achieve the WHO TPP using only 3 genes provides strong evidence for the potential of host-response-based diagnostics to impact clinical practice.

Our analysis has a few limitations. First, we did not have access to the exact published models, or any hyper-parameters used to build the models, for some of the gene signatures compared here. Therefore, we re-implemented these models to the best of our ability by following the details in the corresponding papers. Hence, in the process of trying to build models that reproduced as closely as possible the results reported in the corresponding paper, the choices we made and hyper-parameters we inferred may have been different from the those in the original models. This could have resulted in overfitting, which in turn may have resulted in reduced generalizability of models in independent cohorts and underestimation of their accuracy. We recommend that when diagnostic signatures are published, the corresponding models should be made available, along with a list of hyper-parameters and coefficients to enable reproducibility and comparison between models. Second, for some studies we were not able to use the original training data as they were not available. We chose another dataset that was similar to the discovery cohort described in the corresponding paper. This choice again may have resulted in underestimation of accuracy. Therefore, if a model was extremely sensitive to training data, overfitting may have happened. This limitation points to the need for sharing underlying data used for building a classification model. Third, none of the datasets used in our analysis included patients with nontuberculous mycobacteria infections. Therefore, it is not possible to evaluate whether the signatures compared here can differentiate patients with nontuberculous mycobacteria infections or ATB. Fourth, LTBI was defined using either a tuberculin skin test (TST) or IGRA, which could have different implications for progression to ATB. It is possible that patients identified as having LTBI using different diagnostic criteria could have different transcriptome profiles. Our results showed that a few gene signatures demonstrated consistently high accuracy across datasets irrespective of how LTBI was defined, suggesting that host response to ATB is sufficiently different and robust to overcome the heterogeneity in clinical practice of how LTBI is defined. Importantly, our work described here points to future studies of how existing data could be used to identify differences in the transcriptomes of patients with LTBI diagnosed with TST or IGRA.

Despite the limitations in re-implementation of current ATB diagnostic models, our comparison of 16 blood-based gene signatures strongly suggests the potential of using host-response-based gene signatures for diagnosis of ATB. The fact that a subset of signatures perform with clinically useful accuracy across multiple datasets with no heterogeneity further suggests that they should be explored in larger prospective cohorts for estimating their impact on clinical practice, instead of creating more gene signatures. Further studies should compare these signatures to investigate whether they are correlated with each other (identify the same patients or miss the same patients). If different signatures correctly diagnose different patient populations, it may be advisable to integrate these signatures in a single diagnostic model. However, before validating these signatures in prospective cohorts, they must be published and "locked" for other researchers to investigate such as we have done here. It is highly unlikely that any of these signatures will be measured using RNA sequencing or microarrays in resource-poor areas where TB is prevalent. Therefore, the prospective studies for these signatures should be performed using technologies that are cost-effective when used on a large scale. When host-response-based ATB diagnostics are validated in prospective studies, they should be designed to facilitate the identification of a threshold that can be used in clinical practice. Future prospective trials will also have to understand whether 1 threshold is sufficient across different clinical contexts (e.g., progression from LTBI to ATB, active screening for ATB, and treatment response) or multiple, better-tuned thresholds for each clinical context are needed.

Conclusion

With the increasing number of blood-based signatures for diagnosis of ATB being proposed, it is important to investigate whether measuring host response is appropriate and, if it is, whether any of the existing signatures are able to meet the WHO TPP and should be investigated for translation to clinical practice. We found that when using datasets with only culture-confirmed diagnosis of ATB, only 2 signatures met the minimal WHO TPP for a non-sputum-based triage test. No signature met the minimal WHO TPP when datasets with clinical diagnosis of ATB were included, which may be due to the lower accuracy of clinical diagnoses. Bivariate meta-analysis of these signatures further showed that only 4 out of the 16 gene signatures had no heterogeneity irrespective of which datasets were included in the analysis. Further, we found that 7 signatures met the TPP for a test for predicting progression from LTBI to ATB. Overall, across all comparisons, only the Sweeney3 signature had fewer than 10 genes, met the WHO and FIND TPPs for a non-sputum-based triage test for diagnosis of ATB and predicting progression from LTBI to ATB, and performed robustly with high DOR without heterogeneity and the lowest FPR. We found that higher numbers of genes in a signature did not increase the accuracy of the signature. Our results provide strong evidence for the potential of host-response-based diagnostics in achieving the WHO goal of ending TB by 2035, and should be pursued for clinical implementation.

Supporting information

S1 PRISMA checklist.
(DOCX)

S1 Table. Comparison of re-implemented classification models for 16 gene signatures with the models in the corresponding original reports.
(XLSX)

S1 Text. Description of the re-implemented classification models for each signature.
(DOCX)

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