

AWARD NUMBER: W81XWH-18-1-0253

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

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REPORT DATE: January 2024

TYPE OF REPORT: Final

PREPARED FOR: U.S. Army Medical Research and Development Command
Fort Detrick, Maryland 21702-5012

DISTRIBUTION STATEMENT: Approved for Public Release;
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REPORT DOCUMENTATION PAGE				Form Approved OMB No. 0704-0188	
Public reporting burden for this collection of information is estimated to average 1 hour per response, including the time for reviewing instructions, searching existing data sources, gathering and maintaining the data needed, and completing and reviewing this collection of information. Send comments regarding this burden estimate or any other aspect of this collection of information, including suggestions for reducing this burden to Department of Defense, Washington Headquarters Services, Directorate for Information Operations and Reports (0704-0188), 1215 Jefferson Davis Highway, Suite 1204, Arlington, VA 22202-4302. Respondents should be aware that notwithstanding any other provision of law, no person shall be subject to any penalty for failing to comply with a collection of information if it does not display a currently valid OMB control number. PLEASE DO NOT RETURN YOUR FORM TO THE ABOVE ADDRESS.					
1. REPORT DATE January 2024		2. REPORT TYPE Final		3. DATES COVERED 30Sep2018-29Sep2023	
4. TITLE AND SUBTITLE A Rapid Blood Test to Differentiate Latent Tuberculosis from Active Disease				5a. CONTRACT NUMBER W81XWH-18-1-0253	
				5b. GRANT NUMBER	
				5c. PROGRAM ELEMENT NUMBER	
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				5e. TASK NUMBER	
				5f. WORK UNIT NUMBER	
7. PERFORMING ORGANIZATION NAME(S) AND ADDRESS(ES) THE REGENTS OF THE UNIVERSITY OF CALIFORNIA, SAN DIEGO OFFICE OF CONTRACT & GRANT ADMINISTRATION 9500 GILMAN DR, 0934 LA JOLLA CA 92093-0934				8. PERFORMING ORGANIZATION REPORT NUMBER	
9. SPONSORING / MONITORING AGENCY NAME(S) AND ADDRESS(ES) U.S. Army Medical Research and Development Command Fort Detrick, Maryland 21702-5012				10. SPONSOR/MONITOR'S ACRONYM(S)	
				11. SPONSOR/MONITOR'S REPORT NUMBER(S)	
12. DISTRIBUTION / AVAILABILITY STATEMENT Approved for Public Release; Distribution Unlimited					
13. SUPPLEMENTARY NOTES					
14. ABSTRACT This study aimed to develop a blood-based diagnostic test for tuberculosis (TB) that meets or surpasses WHO Target Product Profiles for a rapid, biomarker-based, non-sputum triage test for active TB. Key activities included optimizing an existing 3-gene mRNA signature predictive of active TB disease, developing a novel 9-gene signature and TB antigen stimulated mRNA signature for improved performance and creating prototype diagnostic cartridges for these assays suitable for deployment in existing Cepheid Xpert platforms. We established clinical sites in Moldova for prospective collection of clinical blood samples for assay development and analytical laboratory testing (N=825) and in Pakistan for prospective clinical field evaluation of Cepheid prototype cartridges (N=500). Results from the assay field performance evaluations are being prepared for publication.					
15. SUBJECT TERMS Tuberculosis, TB, mRNA signature, cartridge, triage test, blood test, finger stick, pre-clinical TB, active TB, latent TB, Moldova, Pakistan, WHO, TPP, biomarkers					
16. SECURITY CLASSIFICATION OF:			17. LIMITATION OF ABSTRACT	18. NUMBER OF PAGES	19a. NAME OF RESPONSIBLE PERSON
a. REPORT	b. ABSTRACT	c. THIS PAGE			USAMRDC
Unclassified	Unclassified	Unclassified	Unclassified	67	19b. TELEPHONE NUMBER (include area code)

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1. INTRODUCTION: *Narrative that briefly (one paragraph) describes the subject, purpose and scope of the research.*

The objective of this research was to develop a TB triage test using blood from a fingerstick that meets or exceeds the WHO Targeted Product Profiles (TPP) for a rapid, biomarker-based, non-sputum triage test for detecting active TB. Our approach involved developing mRNA signatures capable of distinguishing patients with active TB from those with no TB, latent TB, or pre-clinical TB and deploying these signatures in cartridges suitable for near-patient deployment in GeneXpert platforms. We validated prototype signatures using prospectively collected blood samples from TB patients and contacts in the Republic of Moldova. Final signatures were integrated into Cepheid GeneXpert prototype cartridges and field tested with a new cohort of prospectively enrolled patients at risk for TB in Pakistan.

2. KEYWORDS: *Provide a brief list of keywords (limit to 20 words).*

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

3. ACCOMPLISHMENTS: *The PI is reminded that the recipient organization is required to obtain prior written approval from the awarding agency grants official whenever there are significant changes in the project or its direction.*

What were the major goals of the project?

List the major goals of the project as stated in the approved SOW. If the application listed milestones/target dates for important activities or phases of the project, identify these dates and show actual completion dates or the percentage of completion.

The major goal of the project was 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 ($\geq 90\%$ sensitivity when compared with the confirmatory test for active TB (both pulmonary and extrapulmonary) and $\geq 70\%$ specificity against a microbiological reference standard. To accomplish that goal, our specific aims were 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 clinical patients in Pakistan who were at risk of having TB.

The approved SOW also stated site-specific tasks (for UCSD, Moldova, Cepheid, Stanford, the University of Arkansas, and Pakistan) 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 prototype cartridges using blood, finalize prototype cartridge for field testing

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

What was accomplished under these goals?

For this reporting period describe: 1) major activities; 2) specific objectives; 3) significant results or key outcomes, including major findings, developments, or conclusions (both positive and negative); and/or 4) other achievements. Include a discussion of stated goals not met. Description shall include pertinent data and graphs in sufficient detail to explain any significant results achieved. A succinct description of the methodology used shall be provided. As the project progresses to completion, the emphasis in reporting in this section should shift from reporting activities to reporting accomplishments.

1) Major Activities:

- a. *Scientific Collaboration and Discovery:* From project initiation, the UCSD coordinating center facilitated regular communication and scientific collaboration with partners at University of Arkansas, Stanford, and Cepheid. This included establishing quarterly all-team meetings to share progress and barriers, and to ensure alignment and progress. During Year 2, frequency of these discussions increased on an ad hoc basis as necessary to provide opportunities for additional scientific collaboration as signature development and enrollment progressed. Once workflows and critical decision points were established, calls were held between UCSD and each of the collaborating teams on an as-needed basis only. UCSD conducted weekly check-in calls with each of the field sites leading up to, and during, their respective enrollment phases. Each of these meetings were instrumental in fostering a collaborative environment and establishing effective communication channels, which were essential in driving the project toward a successful outcome.
- b. *Enrollment and Data Collection in Moldova:* Participants were enrolled to this prospective field study to complete the aim of validating an improved TB signature. These procedures were carried out in alignment with the protocol approved by the UCSD, DoD, and Moldova Institutional Review Boards. For primary signature analyses, 113 individuals diagnosed with active TB (referred to as index cases) and 454 of these index cases' close contacts were enrolled and blood samples were collected into PAXgene tubes to allow for total RNA extraction upon sample analysis by Stanford. This enrollment was completed to directly address Aim 2 goals, validate the improved TB signature at Stanford.

Two independent enrollment cohorts were recruited following completion of Aim 2 enrollment goals, supported by awarded supplemental funds. The first cohort was enrolled to enrich the pool of clinical samples and to help validate the down-selected signature to be used for Aim 3 field testing of the Cepheid stimulated prototype cartridge. This cohort included 55 microbiologically-confirmed, untreated TB positive patients and 128 contacts, approximately half of whom were uninfected and half of whom had latent TB infection (LTBI) by QuantiFERON reference testing. This supported the preliminary lock of the novel TB antigen stimulated signature developed at Cepheid (discussed in 1d). Following this, an additional 25 active TB patients, 25 LTBI and 25 uninfected participants were enrolled at the Moldova field site in order to de-risk the Aim 3 study in Pakistan by field testing and validating the specific sample stimulation peptides planned for use in the prototype cartridge based on antigen-stimulated blood.

- c. *Signature Development:* In alignment with the study aim to increase the power of the existing prototype 3-gene mRNA signature to discriminate patients with active TB from those with no TB, latent TB or pre-clinical TB, Stanford collaborators completed *in silico* work to develop a novel mRNA signature with 9 genes. Preliminary studies indicated 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). To achieve this, the Stanford team identified and curated gene expression datasets that profiled samples from various bacterial and viral infections and other lung diseases, which combined, enabled an *in silico* analysis of 7,254 samples. The approach used was similar to the approach used when developing the original Stanford 3-gene signature, but with significantly more data. Hundreds of genes were narrowed down to the 9-gene signature by examining performance metrics for discriminating 22 groups of other conditions. Data were compared to *in silico* 3-gene signature performance to analyze improvements. Initial analyses of the 9-gene signature indicated it would meet TPP criteria for distinguishing ATB from healthy controls, but the performance of the signature for distinguishing ATB from latent TB and other diseases was not optimal due to lower-than-expected specificity. Furthermore, collaborators at Cepheid determined that there were technical complications implementing a 9-gene signature within existing cartridge and Xpert hardware environments. The Stanford team began a new discovery analysis for a sub-9-gene signature, but following a rigorous machine learning framework, no other gene combinations could be found that achieved a higher sensitivity and specificity than the 3-gene or 9-gene signatures.

NanoString primers for the 9-gene RNA code set were designed and manufactured, and clinical blood samples collected in Moldova were processed for *in vitro* testing of the 9-gene signature. The *in vitro* testing with clinical samples from the cohort in Moldova indicated that at a minimum sensitivity of 90%, the specificity of the 9-gene signature was 67% while specificity for the 3-gene signature was 57%. Additional studies indicated the 9-gene signature was also highly predictive of treatment failure in patients with drug resistant TB and was highly correlated with PET findings indicating treatment failure. Results from the analysis of the novel 9-gene signature were presented at the Conference for the International Union of Tuberculosis and Lung Disease in 2022, and submitted for peer-review in the journal, *The Lancet Respiratory Medicine*. At the time of this report the journal submitted review comments to the corresponding author and publication is pending.

- d. *Prototype Cartridge Development:* Due to the limitations of the 9-gene signature described above, Cepheid focused prototype cartridge development efforts on deployment of an improved 3-gene signature (they replaced one of the Stanford 3-genes with a gene of their own) and an antigen-stimulated signature cartridge with the potential to differentiate latent TB from active TB and non-TB infected patients. The stimulated cartridge development was a novel product for Cepheid and involved combining two prototype cartridges (one for LTBI detection and one for active TB detection) that were “open” such that they could be run with liquid PCR reagents and with lyophilized reagent beads, which was more similar to a final Cepheid product. The eight markers of the two cartridges were the most promising biomarkers, down selected from a 16-gene candidate set. The objective was to develop GeneXpert assays so that manual procedures of mRNA isolation, cDNA synthesis and dilution and parallel qPCRs could be replaced with a cartridge-based analysis approach. Blood from the cohort in Moldova and other Cepheid studies were used to test the prototype cartridges to determine performance compared to the manual laboratory procedures, and to continue optimization as needed. This was critical to internal prototype verification before procuring the “closed” cartridges for field testing. The two prototype cartridges were merged into one, containing Cepheid mRNA biomarkers, a reference gene and a Cepheid Sample Processing Control (SPC). This resulted in Cepheid’s first 10-color-, 2-signature-, 8-marker-, 2-control prototype cartridge for antigen-stimulated blood. Cepheid implemented a 10-color format version of the prototype compatible with the GeneXpert instrument that launched commercially (CE-IVD) in 2020, instead of a marker configuration compatible with the legacy 6-color version of the current GeneXpert instrument install to allow the option to include more markers with dedicated call-outs/Ct values and increase the chance of success for the two signatures. The prototype cartridge classified

the patients with nearly the same performance as singleplex qPCR using the 8 markers in the cartridge, for both the active TB vs. not infected and for the active TB vs. LTBI signatures, respectively. Cepheid completed development and optimization of the prototype Xpert Latent TB (XLTB) cartridge (stimulated) and the manufactured prototype assays were shipped to the field site. 3-gene Xpert Host Response (XHR; 3-gene) cartridges were also shipped to Pakistan for further field testing.

During cartridge development, Cepheid used the final cohort enrolled in Moldova to validate the specific stimulation peptides used later for antigen-stimulation procedures for the XLTB cartridge testing in Pakistan. Cepheid also compared the stimulation performance of three different antigen tube types to find the optimal stimulation (TB1 from Qiagen, 2-antigen from Lionex, and 3-antigen from Lionex), and ultimately selected the 2-antigen tube from Lionex (based on pragmatic and performance criteria) which was sent to Pakistan for the final XLTB study.

- e. *Prototype Cartridge Field Testing in Pakistan:* Prospective enrollment of patients at risk for TB began during Year 4 of the project and was completed during Year 5. Blood was collected from 500 participants for field testing of both the XHR cartridge and the XLTB cartridge. Due to a slight delay in finalizing and shipping the XLTB cartridges from Cepheid to Pakistan, the first ~40% of blood samples collected for XLTB testing were stimulated and stored frozen before being tested, but the majority of blood samples (~60%) were tested using fresh blood from participants as they were enrolled. Cepheid collaborators were regularly involved in check-ins with the Pakistan field site team and provided quality reviews for data generated from the cartridges. Once enrollment goals were achieved, clinical data were audited and cleaned, and then merged with XHR and XLTB outputs for analysis. UCSF, University of Arkansas, and Cepheid collaborators met to review the preliminary findings for each respective cartridge. At the time of this report, the results of the field testing for each of the cartridges are being prepared for publication.

- 2) **Specific Objectives:** The activities throughout the project supported the specific objectives outlined in the SOW including: scientific collaboration, discovery and augmentation of the 3-gene TB signature, enrollment and blood collection in Moldova, prototype cartridge development and validation, data analysis, and field testing.
- 3) **Significant Results or Key Outcomes:** Significant results of this work included 1) Development of a novel 9-gene mRNA signature for TB diagnosis which was tested both *in silico* and *in vitro* with clinical blood samples, and found to meet WHO TPP performance criteria for a rapid, biomarker-based, non-sputum triage test for detecting active TB disease; 2) Successful enrollment of multiple cohorts of patients and close contacts (N=825) to collect clinical blood samples for mRNA signature testing and validation (Stanford) and cartridge development and validation (Cepheid); 3) Development and analytical testing of a novel prototype cartridge developed by Cepheid to detect active TB and LTBI; 4) Production of high quality clinical field testing performance data for both the XHR and XLTB cartridges (first study of the XLTB cartridge) in a cohort of patients at risk for TB in a setting of intended use in Pakistan.
- 4) **Other Achievements:** This study also successfully implemented QuantiFERON testing for LTBI reference testing in Islamabad, which had not previously been used by the Pakistan National TB Reference Laboratory (NTRL). The current study, along with another identical cohort, provided sufficient sample size for the first population-level estimate of LTBI among patients seeking diagnosis for respiratory diseases in the Islamabad and Rawalpindi areas of Pakistan. The results of this study are being prepared for publication and represent the first in the nation on this topic, and will likely impact future national guidelines on LTBI testing.

- 5) **Stated Goals Not Met:** While the project took longer than expected to complete due to the delays and obstacles resulting from the COVID-19 pandemic, we met and exceeded proposed project goals and aims.

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

If the project was not intended to provide training and professional development opportunities or there is nothing significant to report during this reporting period, state “Nothing to Report.”

Describe opportunities for training and professional development provided to anyone who worked on the project or anyone who was involved in the activities supported by the project. “Training” activities are those in which individuals with advanced professional skills and experience assist others in attaining greater proficiency. Training activities may include, for example, courses or one-on-one work with a mentor. “Professional development” activities result in increased knowledge or skill in one’s area of expertise and may include workshops, conferences, seminars, study groups, and individual study. Include participation in conferences, workshops, and seminars not listed under major activities.

The project provided an opportunity for professional development and advancement of skills for laboratory technicians at the NTRL in Pakistan by providing QuantiFERON training via Qiagen. QuantiFERON testing is not included as part of routine testing at the NTRL, but was included in this research project and will likely be used in the future by the NTRL to evaluate LTBI. This training allowed for technicians to conduct the required testing for the project, but also expanded their technical skill sets which should be a major benefit as Pakistan contemplates shifting to LTBI testing in the near future. Additionally, the UCSD project and ongoing data analysis activities include two NIH T32-funded fellows, one from infectious diseases and one from pediatric infectious diseases. Both fellows are gaining critical training experience working on this project.

How were the results disseminated to communities of interest?

If there is nothing significant to report during this reporting period, state “Nothing to Report.”

Describe how the results were disseminated to communities of interest. Include any outreach activities that were undertaken to reach members of communities who are not usually aware of these project activities, for the purpose of enhancing public understanding and increasing interest in learning and careers in science, technology, and the humanities.

Nothing to Report.

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

If this is the final report, state “Nothing to Report.”

Describe briefly what you plan to do during the next reporting period to accomplish the goals and objectives.

Nothing to Report as this is the final report.

4. **IMPACT:** Describe distinctive contributions, major accomplishments, innovations, successes, or any change in practice or behavior that has come about as a result of the project relative to:

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

If there is nothing significant to report during this reporting period, state “Nothing to Report.”

Describe how findings, results, techniques that were developed or extended, or other products from the project made an impact or are likely to make an impact on the base of knowledge, theory, and research in the principal disciplinary field(s) of the project. Summarize using language that an intelligent lay audience can understand (Scientific American style).

This project made significant strides in the fields of blood-based-biomarker TB diagnostics and big data analysis. Most importantly, the study generated novel development, analytic and field performance data to support continued development and testing of Cepheid's novel antigen-stimulated latent TB cartridge, the XLTB assay for deployment on the existing GeneXpert platform. This prototype tool has the potential to distinguish latent TB infection from active TB, a capability beyond the reach of current TB assays. This study represents the first comprehensive clinical evaluation of the XLTB assay and prototype cartridge on the GeneXpert instrument. Should the field results affirm the laboratory findings, Cepheid could advance this innovative product, potentially transforming the diagnosis of latent and active TB globally.

The study also provided critical field evaluation data for the Cepheid XHR assay and prototype cartridge. The XHR cartridge's continued development and testing in this study represented a pivotal advance toward the implementation of the XHR assay as a commercial product. This cartridge, designed to integrate a refined 3-gene signature (one of the original Stanford genes has been switched out for a Cepheid determined gene), was meticulously engineered to offer accurate diagnosis of active TB disease. While earlier clinical studies mostly focused on performance of the assay in adult patients with TB disease and healthy controls, and showed excellent performance, recent studies of this assay have focused on symptomatic patients at risk for TB, which more accurately reflect the intended use setting for this assay. Unfortunately, in these patient cohorts the assay has shown a reduction in performance to below WHO TPP standards. In our study we focused on recruiting intended use setting patients at risk for TB, but also included adolescents from age 6 through early twenties and obtained unique clinical performance data. Preliminary evidence from our studies indicates the assay meets WHO TPP performance expectations in adolescents while falling slightly below criteria for adults. Given the lack of good TB diagnostic options for adolescents, this could be a major new utility of the assay. The development and validation of the XHR cartridge in our study, therefore, not only reflects a significant scientific achievement in line with existing research but also marks a potential step forward in the global fight against TB.

Dr. Purvesh Khatri from Stanford applied advanced 'big data' techniques and machine learning to existing datasets, enabling him to uncover unique mRNA combinations, or signatures, with an improved capacity to differentiate active TB from LTBI and other diseases. A key achievement was the development of a novel 9-gene signature. This signature is more complex than the previously established 3-gene signature, and demonstrated potential in accurately identifying active TB in patients from diverse global regions in preliminary *in vitro* studies. Moreover, our preliminary analysis of the 9-gene signature revealed its potential superiority over the 3-gene signature. It showed promise in predicting the progression from non-infected or latent TB to active TB up to a year before diagnosis, nearly six months earlier than the 3-gene signature. This groundbreaking finding, if validated in larger studies, could revolutionize the early diagnosis of pre-clinical TB on a global scale. While this novel signature was not implemented as a Cepheid cartridge during the study due to several pragmatic limitations and only marginal performance improvements over the 3-gene prototype cartridge, it remains a signature for future exploitation if its performance can be validated in larger clinical studies.

Additionally, we anticipate that incorporation of QuantiFERON for LTBI diagnosis during this study (not previously utilized in the Pakistan NTRL) could lead to adoption of latent TB testing technology more

routinely in this region. Preliminary results of this testing within the study population indicate that approximately 45% of participants have latent TB infection – which is the first population-level, TB risk-based estimate of LTBI prevalence in Pakistan.

Overall, our work also deepened global understanding of TB infection and disease progression as well as the nuances and complexities of evaluating blood-based biomarkers for TB disease that will have significant impact on future clinical study designs. We used this new knowledge to refine our categorization of TB patients and their contacts, leading to more detailed and effective sample collection and data processing methods. This progress has also opened doors to enhance reference laboratory methods, allowing a more precise depiction of the entire TB disease spectrum.

What was the impact on other disciplines?

If there is nothing significant to report during this reporting period, state “Nothing to Report.”

Describe how the findings, results, or techniques that were developed or improved, or other products from the project made an impact or are likely to make an impact on other disciplines.

The novel data analysis and *in silico* techniques utilized by the Stanford group in this project 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. Additionally, the methods we have adopted for reference sample categorization to help us properly understand the diversity of host biomarker signatures we are observing, while specific to TB in this study, could also have an impact on other diseases with similarly complex, multistage natural histories.

What was the impact on technology transfer?

If there is nothing significant to report during this reporting period, state “Nothing to Report.”

Describe ways in which the project made an impact, or is likely to make an impact, on commercial technology or public use, including:

- *transfer of results to entities in government or industry;*
- *instances where the research has led to the initiation of a start-up company; or*
- *adoption of new practices.*

This project did several things to impact future commercial technologies. Firstly, 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 and people at risk for TB. This project also funded the collection of unique clinical samples and critical data from TB patients and their close contacts in Moldova, which provided a resource for validating early biomarker validation studies. Our methods for clinically categorizing patients acknowledge and incorporate the disease complexities of TB instead of ignoring them. This allowed Cepheid to incorporate the complexity and diversity of host biomarker responses which ultimately supported product design, cost, and implementation.

Within Cepheid, the research and development team made significant progress in several areas that have the potential to be transferred to the product development team 1) Improved design and implementation of the XHR assay in a Cepheid GeneXpert cartridge; 2) Significant new evidence for the clinical performance of the XHR assay in adolescents that might change the product development trajectory of this product; 3) New

knowledge regarding antigen stimulation of blood prior to detecting mRNA expression required for the continued development of the XLTB assay product; 4) The first clinical performance data on the prototype XLTB cartridge that will be used to support continued development of the product.

What was the impact on society beyond science and technology?

If there is nothing significant to report during this reporting period, state “Nothing to Report.”

Describe how results from the project made an impact, or are likely to make an impact, beyond the bounds of science, engineering, and the academic world on areas such as:

- *improving public knowledge, attitudes, skills, and abilities;*
- *changing behavior, practices, decision making, policies (including regulatory policies), or social actions; or*
- *improving social, economic, civic, or environmental conditions.*

With the project identifying over 40% prevalence of LTBI in Islamabad, routine QuantiFERON could be expanded to the population at risk for TB in Pakistan. If this happens, and these patients are identified and treated routinely, the impact on public health in general would be significant.

- 5. CHANGES/PROBLEMS:** *The PD/PI is reminded that the recipient organization is required to obtain prior written approval from the awarding agency grants official whenever there are significant changes in the project or its direction. If not previously reported in writing, provide the following additional information or state, “Nothing to Report,” if applicable:*

Changes in approach and reasons for change

Describe any changes in approach during the reporting period and reasons for these changes. Remember that significant changes in objectives and scope require prior approval of the agency.

Nothing to Report.

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

Describe problems or delays encountered during the reporting period and actions or plans to resolve them.

As reported throughout the project, the impacts of the COVID-19 pandemic were significant for the work of this project. Enrollment in Moldova was halted for many months and RNA extraction work for Stanford’s *in vitro* analyses could not be completed on the anticipated timeline due to laboratory closures. Additional problems that occurred were with shipments to Pakistan due to supply chain disruptions and customs regulations resulting in major delays in deliveries. Despite these large-scale issues, we successfully completed our study objectives as proposed. Although the final analyses and publication of our findings are delayed, we aim to publish the clinical field study results of the XHR and XLTB cartridges in early 2024, with further key analyses from this project expected throughout 2024 and beyond.

Changes that had a significant impact on expenditures

Describe changes during the reporting period that may have had a significant impact on expenditures, for example, delays in hiring staff or favorable developments that enable meeting objectives at less cost than anticipated.

In July 2020, the University of California San Diego replaced its legacy financial management system with Oracle Enterprise Resource Planning (ERP) Cloud. The switchover resulted in major delays in financial updates and reporting, the processing of outgoing sub-award modifications and payments, and real-time adjustments/updates to charges for UCSD personnel/effort. It was anticipated by University leadership that all updates and corrections to the system, and the resolution of all outstanding issues/problems, would be achieved within 12 months; ultimately, there was an extensive transitional phase of nearly 24 months in which financial reporting and processes were negatively affected.

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

Describe significant deviations, unexpected outcomes, or changes in approved protocols for the use or care of human subjects, vertebrate animals, biohazards, and/or select agents during the reporting period. If required, were these changes approved by the applicable institution committee (or equivalent) and reported to the agency? Also specify the applicable Institutional Review Board/Institutional Animal Care and Use Committee approval dates.

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: *List any products resulting from the project during the reporting period. If there is nothing to report under a particular item, state “Nothing to Report.”*

- **Publications, conference papers, and presentations**

Report only the major publication(s) resulting from the work under this award.

- Gupta S, Rao AM, Scott M, Crudu V, Rodwell TC, Catanzaro D, Catanzaro A, Khatri P. A Nine-Gene Blood-Based Signature Meets the World Health Organization Target Product Profiles for Diagnosis of Active Tuberculosis and Predicting Progression from Latent to Active Disease. InC106. FIRE TB LIVES ON 2022 May (pp. A4924-A4924). American Thoracic Society.

Journal publications. *List peer-reviewed articles or papers appearing in scientific, technical, or professional journals. Identify for each publication: Author(s); title; journal; volume: year; page numbers; status of publication (published; accepted, awaiting publication; submitted, under review; other); acknowledgement of federal support (yes/no).*

- Sheng-Wei Pan, Donald G. Catanzaro, Marva Seifert, Rehan R. Syed, Naomi Hillery, Mei-Lin Ho, Valeriu Crudu, Elena Tudor, Nelly Ciobanu, Alexandru Codreanu, Antonino Catanzaro, Timothy C. Rodwell. Predicting stringent QuantiFERON-TB Gold Plus conversions in contacts of tuberculosis patients. *Journal of Microbiology, Immunology and Infection*;56(5):2023;1073-83. <https://doi.org/10.1016/j.jmii.2023.07.014>. **Status:** Published. **Acknowledgement of federal support:** Yes.

- 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. **Status:** Published. **Acknowledgement of federal support:** Yes.
- Sudhasini Panda, Jeffrey Morgan, Catherine Cheng, Mayuko Saito, Robert H. Gilman, Nelly Ciobanu, Valeriu Crudu, Donald G. Catanzaro, Antonino Catanzaro, Timothy Rodwell, Judy S. B. Perera, Teshan Chathuranga, Bandu Gunasena, Aruna D. DeSilva, Bjoern Peters, Alessandro Sette, Cecilia S. Lindestam Arlehamn. Identification of differentially recognized T cell epitopes in the spectrum of tuberculosis infection. Nature Communications;2024;15(1):765. **Status:** Published. **Acknowledgement of federal support:** Yes (NIH).
- Aditya Rao, Sanjana Gupta, Naomi Hillery, Madeleine Scott, Valeriu Crudu, Julio Croda, Timothy Rodwell, Donald G. Catanzaro, Jason Andrews, Antonino Catanzaro, Purvesh Khatri. A nine-gene blood-based signature for predicting progression to and diagnosis of active tuberculosis and monitoring treatment response. **Journal, volume, year, page numbers:** Pending. **Status:** Other (resubmission pending). **Acknowledgement of federal support:** Yes.
- Marva Seifert, Donald G. Catanzaro, Naomi Hillery, Rehan R. Syed, Michael Gracia, Antonino Catanzaro, Sabira Tahseen, Timothy C. Rodwell. Clinical performance of the Cepheid 3-gene host response blood test for identification of tuberculosis in an intended use setting in Pakistan [final title pending]. **Journal, volume, year, page numbers:** Pending. **Status:** Other (in draft, submission expected January 2024). **Acknowledgement of federal support:** Yes.
- Marva Seifert, Donald G. Catanzaro, Naomi Hillery, Rehan R. Syed, Michael Gracia, Antonino Catanzaro, Sabira Tahseen, Timothy C. Rodwell. Clinical performance of a novel prototype GeneXpert latent TB cartridge in patients with respiratory symptoms in Pakistan [final title pending]. **Journal, volume, year, page numbers:** Pending. **Status:** Other (draft pending, submission expected February 2024). **Acknowledgement of federal support:** Yes.

Books or other non-periodical, one-time publications. *Report any book, monograph, dissertation, abstract, or the like published as or in a separate publication, rather than a periodical or series. Include any significant publication in the proceedings of a one-time conference or in the report of a one-time study, commission, or the like. Identify for each one-time publication: author(s); title; editor; title of collection, if applicable; bibliographic information; year; type of publication (e.g., book, thesis or dissertation); status of publication (published; accepted, awaiting publication; submitted, under review; other); acknowledgement of federal support (yes/no).*

Nothing to report.

Other publications, conference papers and presentations. *Identify any other publications, conference papers and/or presentations not reported above. Specify the status of the publication as noted above. List presentations made during the last year (international, national, local societies, military meetings, etc.). Use an asterisk (*) if presentation produced a manuscript.*

Nothing to report.

- **Website(s) or other Internet site(s)**

List the URL for any Internet site(s) that disseminates the results of the research activities. A short description of each site should be provided. It is not necessary to include the publications already specified above in this section.

Nothing to report.

- **Technologies or techniques**

Identify technologies or techniques that resulted from the research activities. Describe the technologies or techniques were shared.

Nothing to report.

- **Inventions, patent applications, and/or licenses**

Identify inventions, patent applications with date, and/or licenses that have resulted from the research. Submission of this information as part of an interim research performance progress report is not a substitute for any other invention reporting required under the terms and conditions of an award.

Under site PI, Dr. Purvesh Khatri, the Stanford team has filed a provisional patent for the 9-gene signature (Application #63183432, Title: METHOD FOR DIAGNOSING ACTIVE TUBERCULOSIS AND PROGRESSION TO ACTIVE TUBERCULOSIS). Approval is pending at the time of this report.

- **Other Products**

Identify any other reportable outcomes that were developed under this project. Reportable outcomes are defined as a research result that is or relates to a product, scientific advance, or research tool that makes a meaningful contribution toward the understanding, prevention, diagnosis, prognosis, treatment and /or rehabilitation of a disease, injury or condition, or to improve the quality of life. Examples include:

- *data or databases;*
- *physical collections;*
- *audio or video products;*
- *software;*
- *models;*
- *educational aids or curricula;*
- *instruments or equipment;*
- *research material (e.g., Germplasm; cell lines, DNA probes, animal models);*
- *clinical interventions;*
- *new business creation; and*
- *other.*

This project contributed to an ongoing specimen collection bank at UCSD. The purpose of this collection is to store clinical samples from consenting research participants to facilitate future research into the diagnosis and treatment of pulmonary and extrapulmonary TB, non-tuberculous mycobacteria, and other diseases.

7. PARTICIPANTS & OTHER COLLABORATING ORGANIZATIONS

What individuals have worked on the project?

UCSD

Antonino Catanzaro	Project PI
Timothy Rodwell	Co-Investigator
Naomi Hillery	Project Manager
Peter Chiles	Laboratory Manager
Laura Myhovich	Project Coordinator

CEPHEID

Jonathan Siegrist	VP Innovation
Malin Nygren	Cepheid Site PI
Ellen Wallace	Assay Development Lead
Sarah Tidstrom	Senior Scientist
Jennie Hermansson	Research Scientist
Antonio Ascue Avalos	Research Scientist
Raquel Rodrigues Palla	Research Scientist
Cherno Sidibeh	Temp. Research Scientist
Aditya Rao	Graduate Student
Madeleine Scott	Graduate Student

INSTITUTE OF PHTHISIOPNEUMOLOGY - MOLDOVA

Valeriu Crudu	Moldova Site PI
Elena Tudor	Clinical Coordinator
Mariana Macari	Administrator
Nelly Ciobanu	Laboratory Coordinator
Alexandru Codreanu	Laboratory Assistant
Eugenia Cula	Disease Investigator
Liudmila Tirsina	Disease Investigator
Nadejda Turcan	Laboratory Assistant
Tatiana Ixari	Laboratory Assistant
Maria Gasco	Laboratory Assistant

NATIONAL TB CONTROL PROGRAM - PAKISTAN

Sabira Tahseen	Pakistan Site PI
Alamdar Hussain Rizvi	Site Project Manager
Faisal Masood	Senior Microbiologist
Uzma Majeed	Site Supervisor
Sajia Ali	Site Supervisor
Faraz Ahmed	Clinical Nurse
Sehrish Aqueel	Clinical Nurse
Faiqa Rasheed	Research Technician
Asfand Khan	Research Technician

STANFORD UNIVERSITY

Purvesh Khatri	Stanford Site PI
Rohit Vashisht	Research Scientist
Hayley Warsinske	Postdoc
Michele Donato	Postdoc
Alex Skrenchuk	Systems Administrator
Hacer Geyicki	PhD Student
Ananth Ganesan	PhD student
Mike Seda	PhD student
Aditya Rao	Graduate Student
Madeleine Scott	Graduate Student

UNIVERSITY OF ARKANSAS

Donald Catanzaro	UARK Site PI
Bill Johnston	Data Core Technician
Chris Angel	Data Core Technician
Maryam Kheirandish Borujeni	Graduate Student
Ahla Ko	Graduate Student

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

If there is nothing significant to report during this reporting period, state “Nothing to Report.”

If the active support has changed for the PD/PI(s) or senior/key personnel, then describe what the change has been. Changes may occur, for example, if a previously active grant has closed and/or if a previously pending grant is now active. Annotate this information so it is clear what has changed from the previous submission. Submission of other support information is not necessary for pending changes or for changes in the level of effort for active support reported previously. The awarding agency may require prior written approval if a change in active other support significantly impacts the effort on the project that is the subject of the project report.

Nothing to report

What other organizations were involved as partners?

If there is nothing significant to report during this reporting period, state “Nothing to Report.”

Describe partner organizations – academic institutions, other nonprofits, industrial or commercial firms, state or local governments, schools or school systems, or other organizations (foreign or domestic) – that were involved with the project. Partner organizations may have provided financial or in-kind support, supplied facilities or equipment, collaborated in the research, exchanged personnel, or otherwise contributed.

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

Organization Name:

National TB Control Program

Location of Organization:

Islamabad, Pakistan

Partner's contribution to project:

- a) Facilities (PI office space, National TB Reference Laboratory [NTRL])
- b) Collaboration

8. SPECIAL REPORTING REQUIREMENTS

See following pages 15-18

9. APPENDICES:

See following pages 19-67



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

PI: Antonino Catanzaro; University of California, San Diego; California **Budget:** \$3,570,150

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

Research Area(s):
Tuberculosis

Award Status: FINAL

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 ($\geq 90\%$ sensitivity when compared with the confirmatory test for active TB (both pulmonary and extrapulmonary) and $\geq 70\%$ 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 mRNA 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 in Pakistan with a new cohort of prospectively enrolled patients suspected to have TB

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. Status: Published.
- Gupta S, Rao AM, Scott M, Crudu V, Rodwell TC, Catanzaro D, Catanzaro A, Khatri P. A Nine-Gene Blood-Based Signature Meets the World Health Organization Target Product Profiles for Diagnosis of Active Tuberculosis and Predicting Progression from Latent to Active Disease. InC106. FIRE TB LIVES ON 2022 May (pp. A4924-A4924). American Thoracic Society.
- Sheng-Wei Pan, Donald G. Catanzaro, Marva Seifert, Rehan R. Syed, Naomi Hillery, Mei-Lin Ho, Valeriu Crudu, Elena Tudor, Nelly Ciobanu, Alexandru Codreanu, Antonino Catanzaro, Timothy C. Rodwell. Predicting stringent QuantiFERON-TB Gold Plus conversions in contacts of tuberculosis patients. Journal of Microbiology, Immunology and Infection;56(5):2023;1073-83. <https://doi.org/10.1016/j.jmii.2023.07.014>. Status: Published.
- Aditya Rao, Sanjana Gupta, Naomi Hillery, Madeleine Scott, Valeriu Crudu, Julio Croda, Timothy Rodwell, Donald G. Catanzaro, Jason Andrews, Antonino Catanzaro, Purvesh Khatri. A nine-gene blood-based signature for predicting progression to and diagnosis of active tuberculosis and monitoring treatment response. Journal, volume, year, page numbers: Pending. Status: Pending resubmission.
- Marva Seifert, Donald G. Catanzaro, Naomi Hillery, Rehan R. Syed, Michael Gracia, Antonino Catanzaro, Sabira Tahseen, Timothy C. Rodwell. Clinical performance of the Cepheid 3-gene host response blood test for identification of tuberculosis in an intended use setting in Pakistan [final title pending]. Journal, volume, year, page numbers: Pending. Status: Other (in draft, submission expected January 2024).
- Marva Seifert, Donald G. Catanzaro, Naomi Hillery, Rehan R. Syed, Michael Gracia, Antonino Catanzaro, Sabira Tahseen, Timothy C. Rodwell. Clinical performance of a novel prototype GeneXpert latent TB cartridge in patients with respiratory symptoms in Pakistan [final title pending]. Journal, volume, year, page numbers: Pending. Status: Other (draft pending, submission expected February 2024).
- Sudhasini Panda, Jeffrey Morgan, Catherine Cheng, Mayuko Saito, Robert H. Gilman, Nelly Ciobanu, Valeriu Crudu, Donald G. Catanzaro, Antonino Catanzaro, Timothy Rodwell, Judy S. B. Perera, Teshan Chathuranga, Bandu Gunasena, Aruna D. DeSilva, Bjoern Peters, Alessandro Sette, Cecilia S. Lindestam Arlehamn. Identification of differentially recognized T cell epitopes in the spectrum of tuberculosis infection. Nature Communications; 2024; 15(1):765. Status: Published.

Patents: Application #63183432, Title: METHOD FOR DIAGNOSING ACTIVE TUBERCULOSIS AND PROGRESSION TO ACTIVE TUBERCULOSIS (provisional patent filed by The Trustees of the Leland Stanford Junior University - Dr. Purvesh Khatri)

Funding Obtained: \$3,570,150

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

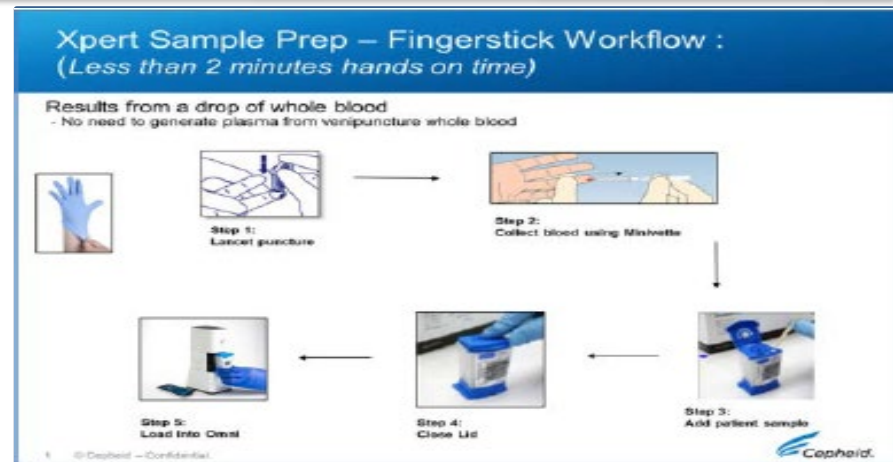
Award Amount: \$3,570,150

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 in Pakistan from clinical patients

Approach

To develop mRNA signatures capable of distinguishing patients with active TB from those with no TB, latent TB, or pre-clinical TB, we applied our computational framework for integrated multi-cohort analysis of gene expression data to pre-collected datasets, utilizing the WHO TB Diagnostics Development framework for test development. We validated prototype cartridge signatures using prospectively collected blood samples from TB patients and contacts in the Republic of Moldova, and field tested the prototype cartridge in an intended-use setting with patients at risk for TB in Pakistan.



Accomplishments: 1) Development of a novel 9-gene mRNA signature meeting WHO TPP performance criteria for a rapid, biomarker-based, non-sputum triage test for active TB; 2) Development and analytical testing of a novel prototype cartridge to detect active TB and LTBI; 3) Implementation of a clinical study of the prototype cartridge in an intended-use setting with patients at risk for TB.

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

CY21-22 Goal – Field Trial

- ✓ Finalize prototype cartridge
- ✓ Field test at clinical site

Budget Expenditure to Date

Projected Expenditure: \$3,570,150

Actual Expenditure: \$3,569,975

Activities	CY	18	19	20	21	22	23
1. Discovery & Augmentation of 3-gene TB signature							
2. Validation							
3. Enrollment & blood collection in Moldova							
4. Prototype cartridge development							
5. Field Trial in Pakistan							
Estimated Budget (\$K)		56,410	376,361	1,258,096	1,131,665	426,089	321,530

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Updated: 1/26/2024

Transition Plan Questionnaire

Directions: Please answer all questions that apply for each product under development. Please fill out one document per product. This is not an application for funding; however, answers will help us understand the outcomes and products from your award.

1. After the award closes, would you be willing to periodically provide voluntary information (via email) regarding the project status (i.e. where the research is headed)? Yes ☒ or No ☐

These responses will help CDMRP demonstrate the return on its investments and will help demonstrate that the CDMRP is a responsible and successful steward of federal research funding.

2. What conclusion(s) does your final data support?

Our final data on the development and evaluation of a Cepheid TB diagnostic prototype product (MTB-HR), based on a 3-gene mRNA expression signature from Stanford, support 3 primary conclusions:

1. The prototype cartridge from Cepheid, run on the existing GeneXpert platform, functioned well with very few indeterminate results or failures
2. The overall performance of the product for diagnosing active TB in patients across all ages did not meet minimum WHO TPP criteria for a non-sputum triage test (90% sensitivity /70% Specificity) compared to reference standards, but could still be useful as the only commercial POC screening product for TB with potentially acceptable performance.
3. When we stratified results by age group, we determined that the performance of the MTB-HR cartridge for diagnosing active TB did meet WHO TPP for a non-sputum triage test in adolescents (6-24 y/o). This is a novel finding, but is supported by the only other large clinical evaluation of the MTB-HR test in pediatric population (Olbrich et al 2024). These findings suggest that the MTB-HR cartridge could become an important product for screening and possibly diagnosing TB in children and adolescents based on a finger stick of blood, which could be a major improvement over current diagnostic alternatives.

3. Will you/have you applied for/obtained follow-on-funding for this project? If yes, please list (a) funding organization, (b) total budget requested/obtained, and (c) title of the funded proposal. This information will be recorded as an outcome to this award.

We have only just analyzed these findings and have not yet applied for follow-on funding, but once the findings are published we will pursue further funding to evaluate the assay performance in adolescent populations.

4. What will be the next step(s) for this project?

1. Complete internal review of the existing draft manuscript of results
2. Submit the manuscript for peer review
3. Work with Cepheid to seek funding for a larger clinical diagnostic study focused on adolescent patients at risk for TB
4. Work with Cepheid on continued product development for this market

5. How would you classify your lead candidate product?

(a) Therapeutic (Small Molecule, Biologic, Cell/Gene Therapy): Please choose, if applicable

(b) Diagnostic

(c) Device

(d) Research Tool to Address a Research Bottleneck

(e) Knowledge Product (Non-material product such as a compound library, database, something that improves clinical practice, education, etc.)

(f) Other - Please Specify:

6. How does your candidate product aid the Warfighter, Veteran, Beneficiary, and/or General Population?

SUPPORTING THE WARFIGHTER The prototype Cepheid MTB-HR test will be deployable as a rapid PoC assay on the existing Cepheid GeneXpert platform. Rapid TB screening, based on a finger stick of blood, even with imperfect sensitivity, could still be a complimentary testing modality to aid in the prompt identification of warfighters in order to initiate more timely treatment, reducing the risk of disease progression and morbidity and mortality associated with advanced disease. It will also limit the potential for disease transmission within military communities. By enhancing the effectiveness of TB screening and control measures, military personnel can maintain optimal health and readiness, ensuring operational effectiveness and mission success.

DUAL-USE CAPACITY. Given the very poor alternatives for TB screening and testing in children and adolescents, an assay with the demonstrated performance MTB-HR test in adolescents could significantly benefit both civilian and military populations for diagnosing TB and monitoring treatment. Replacing sputum testing with a finger-stick blood-based assay for children and adolescents could have a major impact on TB diagnosis in that age group and has the potential to be transformational if the performance we observed can be replicated in a larger multi-country clinical study.

7. Therapy / Product Development, Transition Strategies, and Intellectual Property

Describe the steps and relevant strategies required to move the candidate product (knowledge or tangible) to the next phase of development and/or commercialization. Please address any issues with intellectual property.

PIs are encouraged to explore the technical requirements and the current regulatory strategies involved in product development as well as to work with their organization's Technology Transfer Office (or equivalent regulatory/legal office), federal/international regulatory experts, to develop the transition plan and to explore developing relationships with industry, DoD advanced developers (e.g. USAMMDA), and/or other funding agencies to facilitate moving the product into the next phase.

The IP on which the Cepheid MTB-HR cartridge is based (a 3 Gene mRNA expression signature) was submitted for patent by Stanford prior to this funded project (Patent # US2021/0348233 A1) and licensed for commercial development by Cepheid prior to this project. Cepheid and other funders have invested considerable development resources in developing and testing the prototype MTB-HR cartridge. Early clinical evaluation studies of this prototype product met WHO TPP criteria for sensitivity and specificity, but the subsequent multi-country diagnostic studies failed to meet expected performance criteria, making the continued commercial development of the product less certain. Cepheid has not yet submitted any patents for the MTB-HR product at this time.

A large multi-country diagnostic evaluation study of the product for diagnosing TB in pediatric patients demonstrated a more positive performance that met WHO TPP criteria. This work was just published in February, 2024. Based on that work and our own data (developed in collaboration with Cepheid) demonstrating good performance in adolescent populations, it is possible Cepheid will have renewed interest in commercialization of the product for pediatric and adolescent patients where there a significant medical needs persists.

In addition to the patenting of the 3-gene signature by Stanford (prior to this DoD funded project), Stanford also developed a 9-gene signature during the course of the DoD study. In preliminary laboratory analytical studies, we demonstrated that the 9-gene signature may perform better than the 3-gene signature in adult populations. Stanford filed a provisional patent for this 9-gene signature (iEdison number: 8046501-21-0065). As per report from Stanford, the diagnostic company Inflammatrix took an option to develop a test based on the 9-gene signature, however, given the changes in market conditions, Inflammatrix has decided to not pursue the option and has returned it to Stanford. There are no further plans to license the IP on this test at this time.

The UCSD team has continued to pursue funding to explore the utility and performance of the 9-gene TB signature, most recently in a K08 proposal to NIH (K08AI180348), which was resubmitted to NIH after receiving a good, but not fundable score on the first submission. The UCSD team also continues to communicate with Stanford and seek new funding opportunities to continue development and testing of the 9-gene mRNA signature.

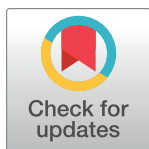
RESEARCH ARTICLE

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

Hayley Warsinske^{1,2}, Rohit Vashisht^{1,2}, Purvesh Khatri^{1,2*}

1 Institute for Immunity, Transplantation and Infection, Stanford University, Stanford, California, United States of America, **2** Center for Biomedical Informatics, Department of Medicine, Stanford University, Stanford, California, United States of America

* pkhatri@stanford.edu



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 ≤ 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

OPEN ACCESS

Citation: Warsinske H, Vashisht R, Khatri P (2019) Host-response-based gene signatures for tuberculosis diagnosis: A systematic comparison of 16 signatures. *PLoS Med* 16(4): e1002786. <https://doi.org/10.1371/journal.pmed.1002786>

Academic Editor: Richard Chaisson, John Hopkins University, UNITED STATES

Received: November 17, 2018

Accepted: March 20, 2019

Published: April 23, 2019

Copyright: © 2019 Warsinske et al. This is an open access article distributed under the terms of the [Creative Commons Attribution License](https://creativecommons.org/licenses/by/4.0/), which permits unrestricted use, distribution, and reproduction in any medium, provided the original author and source are credited.

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](#).

Funding: This work was supported in part by grants from Bill & Melinda Gates Foundation OPP1113682, the NIH/NIAID 1U19AI109662, U19AI057229, and R01AI125197, DoD USAMRAA Award W81XWH-18-1-0253, and by the Clinical and Translational Science Award UL1 RR025744 for the Stanford Center for Clinical and Translational Education and Research (Spectrum)

to P.K. H.C.W. was supported by NIH T32 training grant (5 T32 AI07290-31). The funders played no role in the study design, data collection and analysis, decision to publish, or preparation of the manuscript.

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 Inflammatrix, Inc. Inflammatrix 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.

<https://doi.org/10.1371/journal.pmed.1002786.t001>

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]:

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

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:

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

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

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 <i>Mycobacterium africanum</i>
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.

<https://doi.org/10.1371/journal.pmed.1002786.t002>

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

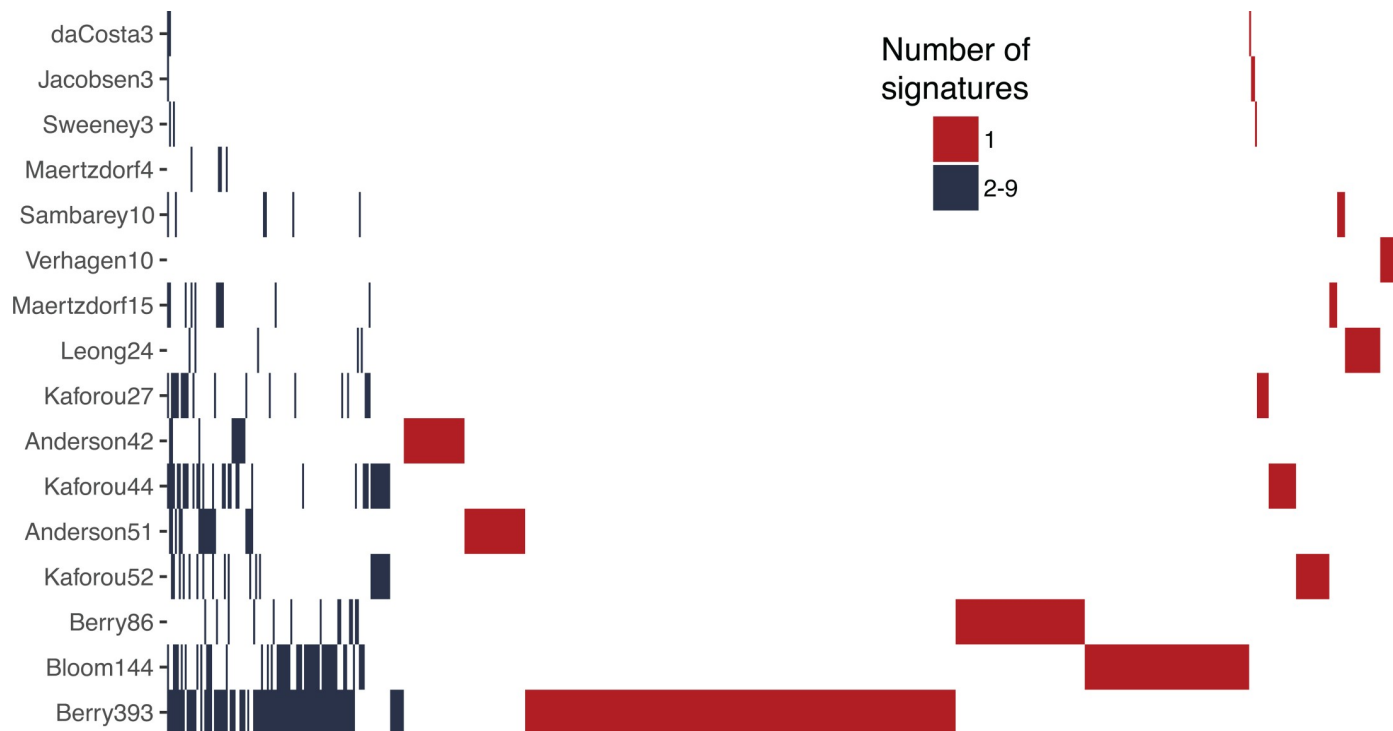


Fig 1. Distribution of genes across the signatures included in this study. Each row represents a gene signature for active tuberculosis diagnosis. Each column represents 1 gene. The number at the end of a signature name represents the number of genes in the given signature. Genes present in only 1 signature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

<https://doi.org/10.1371/journal.pmed.1002786.g001>

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.

<https://doi.org/10.1371/journal.pmed.1002786.t003>

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.

<https://doi.org/10.1371/journal.pmed.1002786.t004>

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.

<https://doi.org/10.1371/journal.pmed.1002786.t005>

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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Original Article

Predicting stringent QuantiFERON-TB Gold Plus conversions in contacts of tuberculosis patients



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Received 15 March 2023; received in revised form 23 July 2023; accepted 31 July 2023

Available online 4 August 2023

KEYWORDS

Interferon-gamma
release assay;
QuantiFERON-TB Gold
plus;
Stringent conversion;
Tuberculosis contacts

Abstract *Objectives:* To assess associations between disease severity in index TB patients and QuantiFERON-TB Gold Plus (QFT-Plus) results in contacts, and predictors for QFT-Plus conversion in contacts over 6–12 months.

Methods: TB patients ($n = 295$) and the contacts ($n = 1051$) were enrolled during 2018–2021 with QFT-Plus performed at baseline and months 6 and 12. A strong CD8 response was defined as TB2 interferon gamma (IFN- γ) response minus TB1 >0.6 IU/ml and stringent conversion as change from QFT-plus negative to high-positive QFT-Plus (TB1 or TB2 IFN- γ responses >0.7 IU/ml).

Results: Contacts with index TB patients with sputum smear $>1+$ was associated with positive QFT-Plus compared to those without ($p < 0.001$). Contacts with index TB patients with bilateral lung disease were more likely to have strong CD8 responses than those without ($p = 0.038$). QFT-Plus stringent conversion occurred in 9.7% of contacts over 6–12 months.

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A TB1 IFN- γ response ≥ 0.03 IU/ml combined with a TB2 ≥ 0.06 IU/ml was predictive of a 19-fold increased risk for QFT-Plus stringent conversion in contacts (odd ratio 19.565 [8.484–45.116], $p < 0.001$).

Conclusion: Bacterial burden and bilateral lung disease of index TB patients were associated with positive QFT-Plus and strong CD8 responses in contacts. TB1 and TB2 IFN- γ responses were synergistically predictive of stringent conversion in contacts.

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Introduction

It is estimated that one-third of the world's population has been infected with *Mycobacterium tuberculosis* (MTB).¹ Although most people with MTB infection live healthy lives with a latent tuberculosis infection (LTBI), they carry a 5–10% lifelong probability of developing active tuberculosis (TB),² with an incidence of 0.84–3.3 per 1000 person-years, depending on exposure timeline and risk factors.^{3,4} Half of that risk of TB disease occurs in the first few years after exposure, suggesting a clinical need for increasing prophylactic treatment for LTBI in contacts to decrease the probability of progressing to active TB.^{5,6} Interferon-gamma (IFN- γ) release assays (IGRA) are the *in vitro* diagnostic standard for LTBI diagnosis, but due to limitations in their ability to differentiate LTBI from active TB disease, or to predict LTBI patients most likely to convert to active TB disease, their clinical potential has not been fully realized.

QuantiFERON-TB Gold Plus (QFT-Plus, Qiagen, Hilden, Germany) is the fourth generation of IGRA tests developed by Qiagen and uses a peptide cocktail of MTB ESAT-6 and CFP-10 proteins to stimulate T cells and report a quantitative IFN- γ response from patient blood *in vitro*. QFT-Plus measures the IFN- γ response from CD4⁺ T cells in its TB Antigen Tube 1 (TB1) and IFN- γ response from both CD4⁺ and CD8⁺ T cells in its TB Antigen Tube 2 (TB2).⁷ It is thought that the difference of IFN- γ between TB2 and TB1 tube (TB2-TB1) can be used as a proxy for CD8 response.⁸ A high CD8 IFN- γ response is broadly indicative of recent MTB infection and has been correlated with active TB disease.^{8–11}

Previous versions of the QFT assay have been used to assess the risk of active TB in contacts and while the QuantiFERON Gold In-Tube (QFT-GIT) did not evaluate CD8 response, smear-positivity and severe lung disease of index TB patients was associated with the risk of TB in contacts^{10,12–14} and high QFT-GIT results in contacts at baseline were moderately predictive of incident TB disease.^{15,16} Additionally, contacts have been shown convert from QFT-GIT negative at baseline to strong positive during follow-up,¹⁷ suggesting the potential of monitoring LTBI status to prioritize contacts. A large longitudinal study assessing serial IFN- γ responses using the QFT-GIT test in a high TB burden area, determined that a IFN- γ conversion from extremely negative to high-positive QFT-GIT (IFN- γ > 0.70 IU/ml) at day 360 was predictive of a 10-fold increased risk of developing TB over 2 years.¹⁸ Similar studies have not been completed by using QFT-Plus to assess stringent

conversions during follow-up and risk factors of conversion have not been explored comprehensively. Filling in this knowledge gaps on QFT-Plus is clinically relevant because appropriate interpretation of IGRA results may help guide precision management for contacts, especially for those with initial QFT-Plus negative (IFN- γ < 0.35 IU/ml), suggesting no initial mandatory therapy.

In our study, we used a prospective clinical study in TB patients and their contacts to examine the association between the disease severity of index TB patients and the QFT-Plus IFN- γ responses in contacts, with a specific focus on the contributions of the CD8 response. In addition, we evaluated the QFT-Plus results from contacts over 12-month to examine the temporal correlations between baseline factors and QFT-Plus stringent conversion among contacts who initially tested QFT-Plus negative.

Methods

Study design and enrollment

This prospective cohort study was conducted in partnership with the Republic of Moldova's National TB Program (NTP) encompassing their TB clinics, contact tracing program, and the National TB Reference Laboratory (NTRL). This study was approved by the Ethics Committee of the Chiril Draganiuc Institute of Phthisiopneumology in the Republic of Moldova, the University of California San Diego Human Research Protections Program (Project #180068), the University of Arkansas Institutional Review Board (Protocol #1806126401R002), and the Department of Defense Office of Human Research Oversight (Project #E00212.1a) in the US.

Patients with active pulmonary TB and their contacts were enrolled from October 2018 through October 2021. New TB patient identification was carried out by staff monitoring Moldova's electronic NTP System of Information for Monitoring and Evaluation of TB (SIME-TB) and contact investigations were carried out by district TB nurses. New TB patients identified for public health contact tracing activities and their contacts were approached to determine their interest in the study and enrolled following informed consented.

The inclusion criteria for participants with pulmonary TB were (1) sputum acid-fast bacilli (AFB) smear-positive or nucleic acid amplification test (NAAT)-positive within the previous four weeks, (2) ≥ 18 years old, and (3) willing to

identify contacts in the past three months, whether living with the contacts in the same house or not. TB patients were excluded from the study if they had received any TB treatment for more than 4 weeks prior to screening and 12 weeks prior to testing. The inclusion criteria for contacts were (1) exposure to a smear-positive or NAAT-positive TB patient, (2) ≥ 5 years old and ≥ 16 kg, (3) no evidence of TB on clinical evaluation which includes chest x-ray. Contacts were excluded if they (1) were pregnant, (2) were ever treated to prevent TB (therapy for LTBI is not routinely provided in the Republic of Moldova), (3) had a history of prior TB disease, (4) had their QFT-Plus tests after 12 weeks post exposure, or (5) had no QFT-Plus at baseline. Contacts identified to have active TB disease within 3 months after presumed exposure were classified as co-prevalent TB patients in the analysis.

QFT-Plus assay and measurement

Ten milliliters of whole blood were drawn from each participant into a Lithium Heparin tube upon enrollment and at 6 and 12-months following enrollment. All blood samples were transported daily by dedicated vehicle to the NTRL within 8 h at room temperature, where blood was transferred into the four QFT-Plus tubes and IFN- γ levels were measured per the manufacturer's instruction. QFT-Plus results were classified according to definitions in Table 1, defining stringent conversion as QFT-Plus change from QFT-Plus negative (both TB1 and TB2 IFN- γ responses <0.35 IU/ml) to high-positive (either TB1 or TB2 IFN- γ responses ≥ 0.70 IU/ml) and strong CD8 response as TB2-TB1 IFN- γ >0.6 IU/ml. Demographic data on age, sex and comorbidities were collected. For TB patients, sputum AFB smear grade, culture results, and the presence of bilateral lung disease and/or cavitory lung lesion were recorded.

Statistical analysis

Student's *t* test and Mann–Whitney *U* test were used to compare group means and medians as appropriate and the χ^2 test or Fisher's exact test was used to compare proportions. A paired *t* test was performed to compare TB1 and

TB2 IFN- γ responses within groups. Receiver operating characteristic (ROC) analysis was used to analyze the ability of TB1 and TB2 to predict stringent conversion of QFT-Plus, areas under the ROC curve (AUCs) and the maximum value of the Youden index were calculated to determine the optimal cut-off values.¹⁹ Logistic regression analysis was used to identify factors associated with QFT-Plus stringent conversion at months 6 and 12. Odds ratios (ORs) and 95% confidence intervals (CIs) were generated. Kaplan–Meier survival curve with log-rank test was used to examine the differences in probability of QFT-Plus conversion between groups stratified by baseline TB1 and TB2 IFN- γ responses. Analyses were conducted by SPSS (v18.0; SPSS Inc, IL, USA) with figures generated by GraphPad Prism (v6.0; GraphPad Software Inc, CA, USA) and Sankey Diagram tool (<https://sankeymatic.com/build/>).

Results

Characteristics of participants

Over the 3-year period, 295 TB patients and 1051 contacts were enrolled (Fig. 1). Nearly all TB patients (97.9%) were NAAT- or culture-positive for MTB. The mean age of TB patients and contacts were similar (43.9 ± 12.6 vs 42.6 ± 17.3 years) but 10.6% of contacts were under 18 years old. Notably, 77% of contacts had exposure to index TB patients that were AFB smear-positive and 40% to those with AFB $>1+$ (Table 2, also see Supplementary Table S1 for detailed data). After excluding participants with indeterminate QFT-Plus results, 72% of TB patients ($n = 204$) and 35% of contacts ($n = 361$) were positive QFT-Plus ($p < 0.001$).

Clinical factors and QFT-Plus at baseline

In TB patients, TB2 IFN- γ response was significantly higher than TB1 ($p = 0.003$). In contacts, there were no significant differences between TB1 and TB2 responses. Both TB1 and TB2 results were higher in TB patients than in contacts ($p < 0.001$) (Fig. 2A). The CD8 response was higher in TB patients than in contacts ($p < 0.001$) (Table 2). In positive

Table 1 Classification and definition of QuantiFERON-TB Gold Plus (QFT-Plus) results in this study.

Classification	Definition
TB1 IFN- γ response	Interferon gamma (IFN- γ) level in TB1 antigen tube minus that in nil tube (TB1-nil)
TB2 IFN- γ response	IFN- γ level in TB2 antigen tube minus that in nil tube (TB2-nil)
QFT-Plus negative	Both TB1-nil and TB2-nil IFN- γ levels <0.35 IU/ml or $<25\%$ of nil value ^a
Positive QFT-Plus	Either TB1-nil or TB2-nil IFN- γ level >0.35 IU/mL and $>25\%$ of nil value ^b
Low-positive QFT-Plus	Highest level of TB1-nil and TB2-nil IFN- γ between 0.35 and 0.69 IU/ml ^b
High-positive QFT-Plus	Highest level of either TB1-nil or TB2-nil IFN- γ ≥ 0.70 IU/ml ^b
CD8 IFN- γ response	TB2 IFN- γ response minus TB1 (TB2-TB1)
Strong CD8 response	TB2-TB1 IFN- γ level >0.6 IU/ml
QFT-Plus stringent conversion	On follow-up, change from QFT-Plus negative to high-positive QFT-Plus
Indeterminate QFT-Plus	1) IFN- γ level in nil tube >8.0 IU/ml or 2) IFN- γ level in mitogen tube <0.5 IU/ml when TB1-nil and TB2-nil IFN- γ levels were <0.35 IU/ml or was $<25\%$ of nil value

^a Along with interferon gamma level in mitogen tube ≥ 0.5 IU/ml and that in nil tube ≤ 8.0 IU/ml.

^b Along with interferon gamma level in nil tube ≤ 8.0 IU/ml.

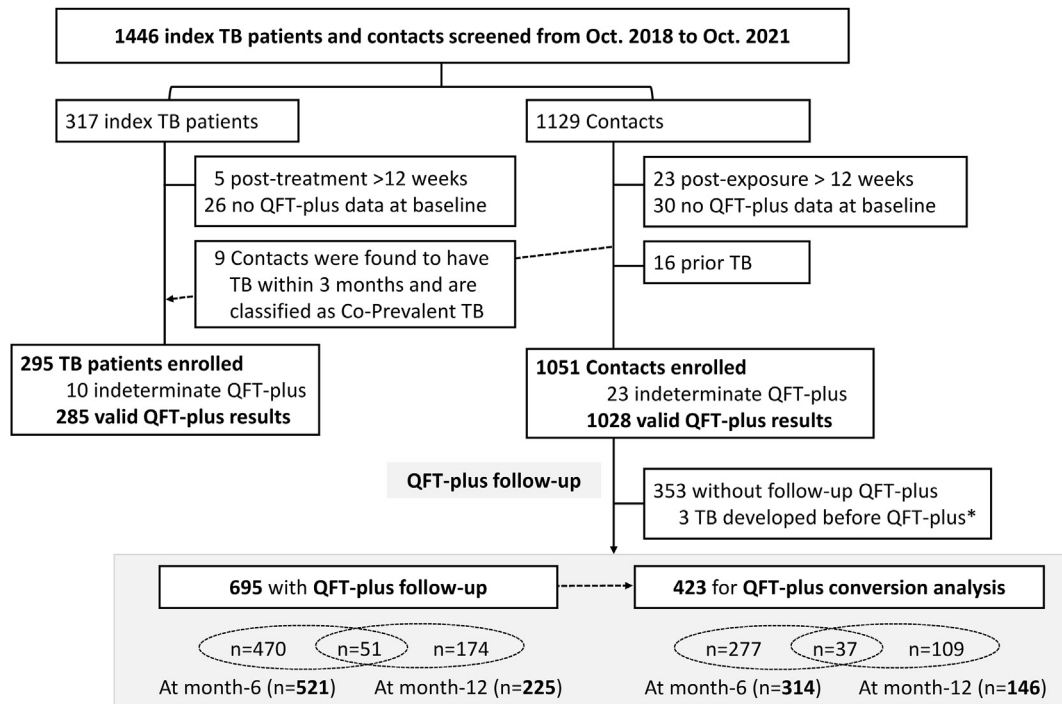


Figure 1. Flow chart of enrollment. Follow-up QFT-Plus data were available for 695 contacts: 521 tested at 6 months (median 6.5 months [IQR 6.0–7.5]) and 225 at 12 months (median 13.2 months [11.8–14.7]), including 51 at both time points. *During follow-up, 16 contacts were known to have microbiologic confirmed TB after the initial 3 months: 5 with lost to follow-up for QFT-Plus, 8 with follow-up QFT-Plus and TB development later, and 3 with TB developed before QFT-Plus. The last 3 ones were excluded from analysis.

QFT-Plus population, CD8 response was also higher in TB subgroup than in contact subgroup (0.30 ± 1.44 vs -0.06 ± 0.75 , $p = 0.001$) although both TB1 and TB2 IFN- γ response were not statistically different between the subgroups (Fig. 2A). The proportion of strong CD8 response was 15% in TB patients, which was higher than the 3% in all contacts ($p < 0.001$) and the 9% in the contacts with positive QFT-Plus (i.e., contacts with LTBI) ($p = 0.026$). In positive QFT-Plus population, a strong CD8 response was associated with a 2.66-odds for TB diagnosis versus LTBI (95% CI 1.62–4.34, $p < 0.001$).

Contacts of TB patients with AFB $>1+$ had higher TB1 and TB2 IFN- γ responses than contacts of TB patients with AFB $\leq 1+$ ($p = 0.012$ and $p = 0.022$) and contacts of TB patients with AFB $>1+$ were more likely to have a positive QFT-Plus than contacts of index patients without (46.2% [146/316] vs 31.4% [154/491]) ($p < 0.001$). Moreover, contacts of TB patients with bilateral lung disease had higher TB2, but not TB1, IFN- γ response than contacts of TB patients without bilateral lung disease ($p = 0.046$) (Fig. 2B). Contacts of index patients with bilateral lung disease were also more likely to have strong CD8 responses than contacts of index patients without (4.7% [20/430] vs 2.1% [9/438]) ($p = 0.038$) (Fig. 2C).

Longitudinal QFT-Plus results among contacts

Contacts with follow-up QFT-Plus data ($n = 695$) were older than those without (43.6 ± 17.4 vs 40.7 ± 16.8 years,

$p = 0.011$) but the male proportion was not significantly different (41% vs 46%, $p = 0.165$). Among contacts with initial high-positive QFT-Plus, 70% of contacts ($n = 89$) had persistent high-positive QFT-Plus results at 6 months and 92% ($n = 47$) were high-positive QFT-Plus at 12 months (Fig. 3A–B). Importantly, among contacts with initial QFT-Plus negative results, over 6% of contacts ($n = 20$) had stringent conversions by 6 months and 14% ($n = 21$) had stringent conversions by 12 months, with a total of 10% of them having QFT-Plus stringent conversion over 6–12 months ($n = 41/423$).

Factors associated with stringent conversion

Baseline TB1 and TB2 IFN- γ responses were higher in contacts with stringent conversion at month 6 and 12 than those without (all $p < 0.001$) while CD8 responses were not different in any subgroup (Table 3). The smear status and lung lesions of index TB patients were not associated with stringent conversion. In ROC curve analyses, the AUC for using baseline TB1 and TB2 IFN- γ responses to predict stringent conversion at month 6 were 0.793 (95% CI, 0.672–0.913) and 0.790 (95% CI, 0.686–0.895) with predictions slightly higher for conversion at 12 months with AUCs of 0.865 (95% CI, 0.789–0.940) and 0.854 (95% CI, 0.766–0.941). The optimized threshold values for prediction at month 6 was ≥ 0.03 IU/ml for TB1 and ≥ 0.06 IU/ml for TB2 with similar threshold values for month 12 follow ups (Table 3). The sensitivity and specificity of using TB1

Table 2 Demographic data of patients with pulmonary tuberculosis (TB) and TB contacts (n = 1346).

Variables	TB patients (n = 295)	Contacts (n = 1051)	Contacts subgroups by baseline QFT-Plus results		
			Positive (n = 361)	Negative (n = 667)	IDT (n = 23)
Age, years (n = 1337) ^a	43.9 ± 12.6	42.6 ± 17.3	43.4 ± 17.9	42.3 ± 16.8	37.6 ± 21.2
Male sex (n = 1340)	224 (76%)	447 (43%)***	153 (43%)	285 (43%)	9 (39%)
Ever smoker (n = 1249)	179 (63%)	217 (22%)***	71 (22%)	141 (23%)	5 (24%)
HIV positive (1142)	12 (4%)	8 (1%)**	3 (1%)	5 (1%)	0
Diabetes mellitus (1136)	11 (4%)	24 (3%)	8 (3%)	16 (3%)	0
Clinical presentation of TB patients					
Smear positive (n = 280)	210 (75%)				
Smear grade >1+ (n = 280)	108 (39%)				
Bilateral lung disease (n = 295)	159 (54%)				
Cavitary lung lesion (n = 295)	90 (31%)				
Characteristics of index TB patients					
Smear positive (n = 827)		640 (77%)	242 (81%)	381 (75%)	17 (85%)
Smear grade >1+ (n = 827)		328 (40%)	146 (49%)	170 (34%)***	12 (60%)
Bilateral lung disease (BLD, n = 891)		442 (50%)	169 (54%)	261 (47%)	12 (52%)
Cavitary lung lesion (CLD, n = 891)		280 (31%)	102 (32%)	173 (31%)	5 (22%)
BLD and/or CLD (n = 891)		500 (56%)	189 (60%)	299 (54%)	12 (52%)
QFT-Plus results at M0 (n = 1313)^b	(n = 285)	(n = 1028)	(n = 361)	(n = 667)	
Positive QFT-Plus	204 (72%)	361 (35%)***	361 (100%)	0***	
High-positive QFT-Plus	153 (54%)	275 (27%)***	275 (76%)	0***	
TB1-nil INF-γ (IU/ml)	1.58 ± 2.25	0.79 ± 1.79***	2.23 ± 2.40	0.00 ± 0.30***	
TB2-nil INF-γ (IU/ml)	1.80 ± 2.49	0.76 ± 1.76***	2.17 ± 2.36	0.00 ± 0.31***	
CD8 IFN-γ response (IU/ml)	0.22 ± 1.23	−0.02 ± 0.45**	−0.06 ± 0.75	−0.00 ± 0.07**	
Strong CD8 response	43 (15%)	33 (3%)***	33 (9%)	0***	
QFT-Plus results at M6 (n = 493)^c		(n = 493)	(n = 165)	(n = 314)	(n = 14)
Positive QFT-Plus		182 (37%)	137 (83%)	35 (11%)***	9 (64%)
High-positive QFT-Plus		134 (27%)	108 (65%)	20 (6%)***	5 (36%)
TB1-nil INF-γ (IU/ml)		0.77 ± 1.89	2.12 ± 2.71	0.16 ± 0.64***	0.94 ± 1.08
TB2-nil INF-γ (IU/ml)		0.77 ± 1.78	1.93 ± 2.59	0.16 ± 0.60***	0.91 ± 1.13
CD8 IFN-γ response (IU/ml)		−0.06 ± 0.52	−0.19 ± 0.83	0.00 ± 0.23*	−0.03 ± 0.29
Definite QFT-Plus results at M12 (n = 224)^d		(n = 224)	(n = 77)	(n = 146)	(n = 1)
Positive QFT-Plus		105 (47%)	72 (94%)	31 (21%)***	1 (100%)
High-positive QFT-Plus		90 (40%)	67 (87%)	21 (14%)***	1 (100%)
TB1-nil INF-γ (IU/ml)		1.65 ± 2.83	4.13 ± 3.27	0.35 ± 1.33***	1.72
TB2-nil INF-γ (IU/ml)		1.64 ± 2.80	4.20 ± 3.28	0.30 ± 1.11***	1.54
CD8 IFN-γ response (IU/ml)		−0.01 ± 1.03	0.06 ± 1.63	−0.04 ± 0.47	−0.18

^a For detailed information on missing data, please refer to [Supplementary Table S1](#).^b Ten TB patients and 23 contacts with indeterminate QFT-Plus results at baseline (M0) were excluded from analysis.^c Among 521 contacts with follow-up QFT-Plus test at month 6 (M6), 28 with indeterminate results were excluded from analysis.^d Among 225 contacts with follow-up QFT-Plus test at month 12 (M12), only one with indeterminate result was excluded from analysis.

Continuous and categorical data are expressed as mean ± standard deviation (SD) and number (%).

IDT indicates indeterminate; QFT-Plus, QuantiFERON-TB Gold Plus; TB1-nil, the difference of Interferon-gamma (IFN-γ) between tube with TB1 antigen and without stimulation (nil); TB2-nil, the difference of IFN-γ between tube with TB2 antigen and without stimulation (nil); TB2-TB1, the difference of IFN-γ between tube with TB1-antigen and tube with TB2-antigen stimulation.

* indicates $p < 0.05$, ** $p < 0.01$ and *** $p < 0.001$ for comparisons between index TB patients and contacts and between contacts with positive QFT-Plus and with QFT-Plus negative results.

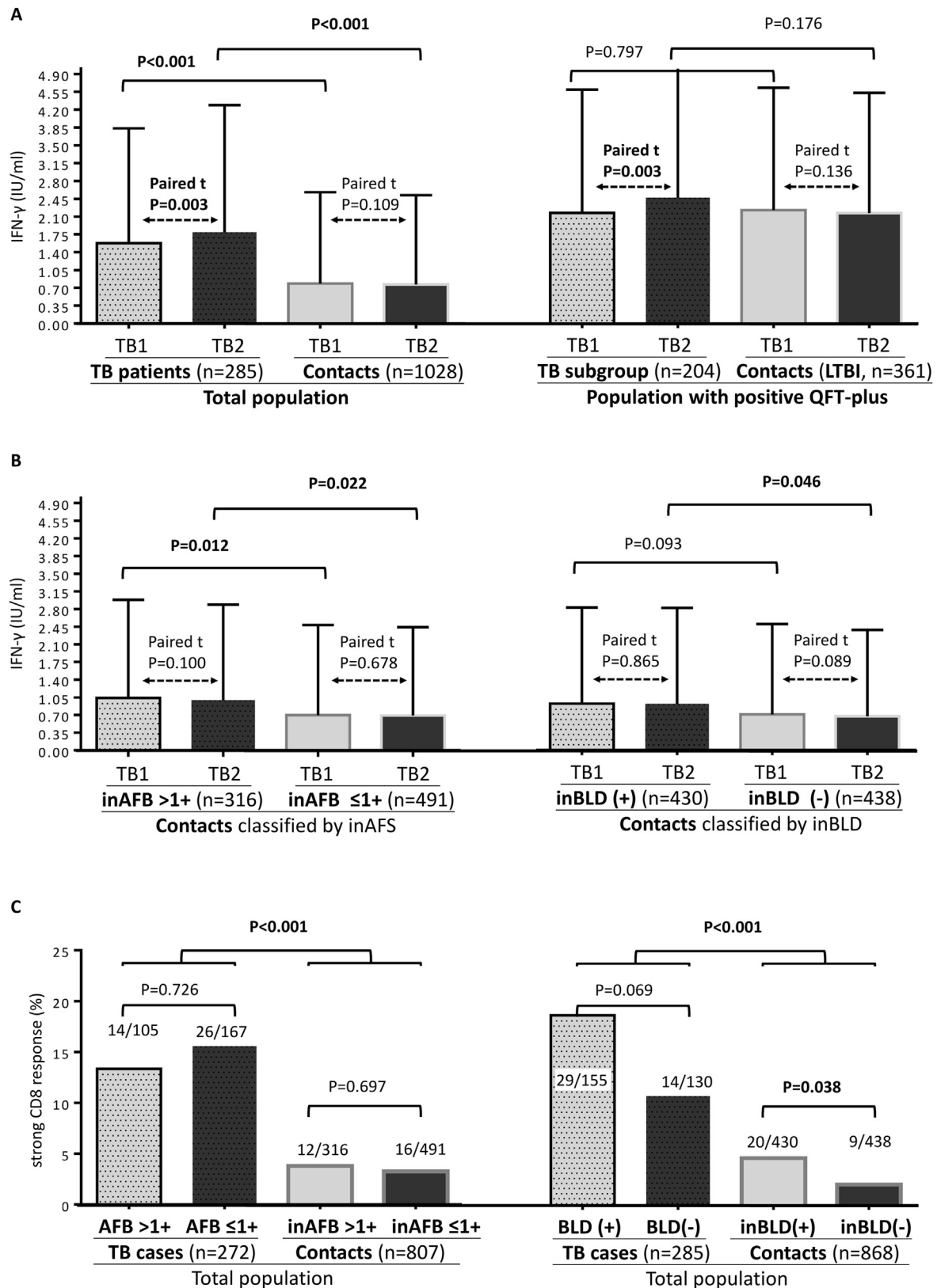


Figure 2. (A) QuantiFERON Gold Plus (QFT-Plus) TB Antigen Tube 1-nil (TB1) and TB Antigen Tube 2-nil (TB2) interferon gamma (IFN- γ) responses in TB patients and contacts as well as IFN- γ responses in positive QFT-Plus TB patients and positive QFT-Plus contacts (contacts with latent TB infection). (B) TB1 and TB2 IFN- γ responses in contacts subgroups classified by index factors were compared. The factors were sputum acid-fast bacilli (AFB) smear grade of index case (inAFB) and bilateral lung disease (BLD) of index TB patients (inBLD). Of note, inAFB >1+ was correlated with initial positive QFT-Plus (odds ratio [OR] 1.879 [95%

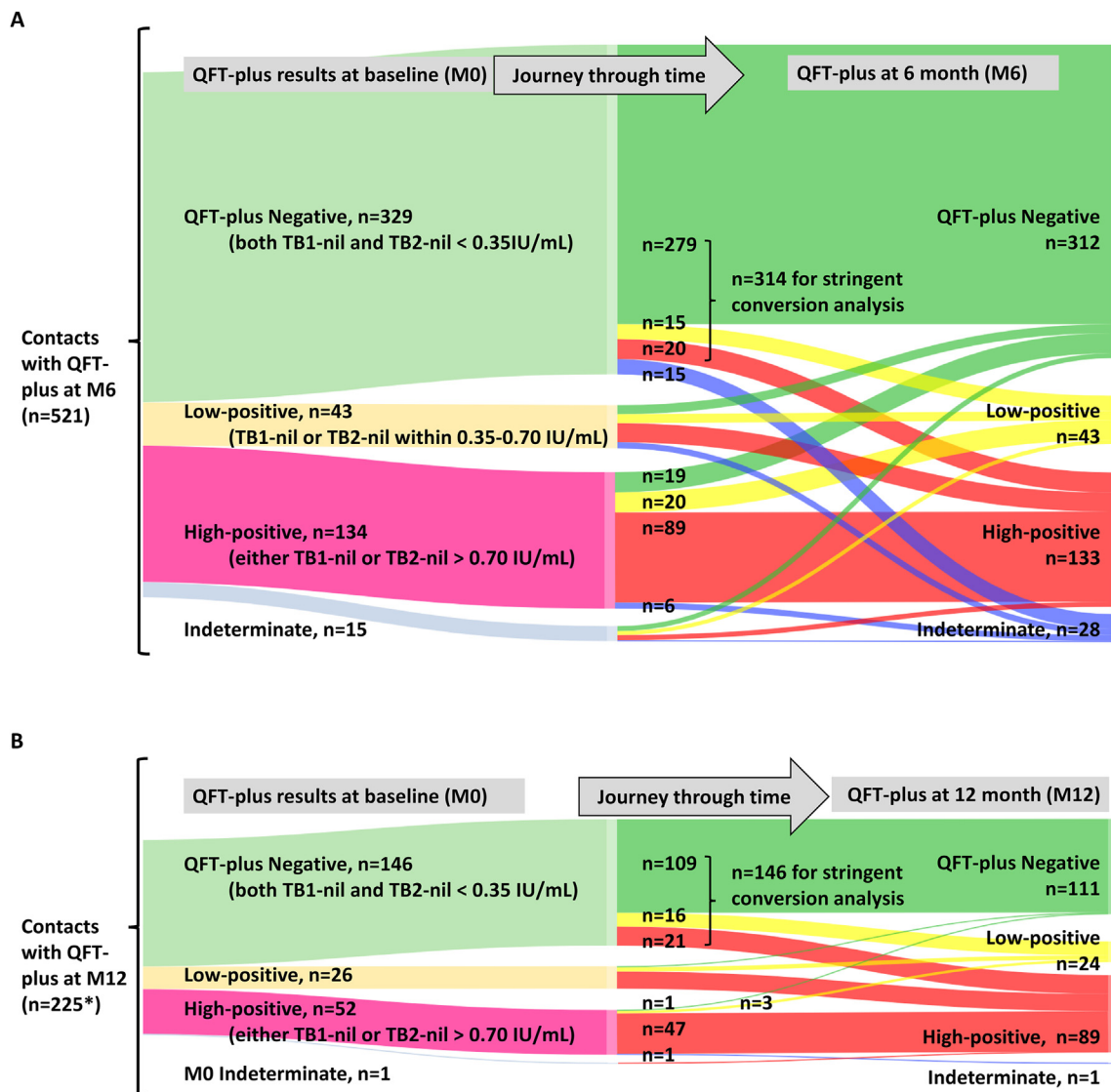


Figure 3. Journey of QFT-Plus status of contacts from baseline (M0) to follow-up at months 6 (M6) (A) and months 12 (M12) (B). *Among contacts with follow-up QFT-Plus at both 6 and 12 months, 37 were initially QFT-negative: 31 (83.7%) of them were always QFT-Plus negative at both time points, 5 (13.5%) moved from persistent negative at months 6 to high-positive at months 12 (QFT-Plus stringent conversion), and 1 (2.7%) from stringent conversion status at month 6 to negative reversion at month 12.

≥ 0.03 and TB2 ≥ 0.06 IU/ml to predict stringent conversion was 0.75 and 0.81 for 6 months and 0.78 to 0.85 for 12 months (see [Supplementary Table S2](#)).

In a logistic regression analysis ([Table 4](#)), contacts with TB1 ≥ 0.03 and TB2 ≥ 0.06 IU/ml at baseline had an increased risk of QFT-Plus stringent conversion at month 6 (adjusted OR 17.601 [5.520–56.121], $p < 0.001$) which was even higher at month 12 (adjusted OR 23.642 [6.752–82.782], $p < 0.001$). In a Kaplan–Meier curve analysis ([Fig. 4](#)), the cumulative probability of QFT-Plus stringent conversion

during follow-up was exponentially higher in contacts with TB1 IFN- $\gamma \geq 0.03$ plus TB2 ≥ 0.06 IU/ml ($n = 94$, 22.2%) than in the other two groups (both $p = 0.001$). While TB1 ≥ 0.03 IU/ml alone was associated with a 14.4-fold increased risk of stringent conversion (adjusted OR 14.389 [95% CI 6.345–32.627], $p < 0.001$) in contacts over a 6–12-month period, TB1 IFN- $\gamma \geq 0.03$ plus TB2 ≥ 0.06 IU/ml was correlated with a 19.6-fold increased risk for stringent conversion after adjustment for age and sex (adjusted OR 19.565 [8.484–45.116], $p < 0.001$) ([Table 4](#)).

confidence interval 1.403–2.517], $p < 0.001$) and high-positive QFT-Plus in contacts (OR 1.674 [1.229–2.281], $p = 0.001$) but it was not associated with strong CD8 responses in contacts. (C) The proportions of strong CD8 response (TB2-TB1 IFN- $\gamma \geq 0.60$ IU/ml) were showed in TB patients classified by related AFB and BLD as well as in contacts by inAFS and inBLD. Notably, inBLD was associated with strong CD8 response in contacts (OR 2.325 [1.047–5.166], $p = 0.038$) but was not correlated with positive QFT-Plus. In TB patients, the presence of BLD, but not AFB status, was marginally associated with strong CD8 response.

Table 3 Characteristics of initially QFT-Plus negative contacts with stringent conversion (SC) and without SC during 6- and 12-month follow-up.

Variables	QFT-Plus at month-6 (n = 314) ^a		P value	QFT-Plus at month-12 (n = 146) ^b		P value
	With SC (20)	Without SC (n = 294)		With SC (n = 21)	Without SC (n = 125)	
Age, years	40.1 ± 14.0	43.3 ± 17.3	0.411	44.4 ± 11.9	44.2 ± 16.6	0.944
Male sex	8 (40%)	114 (39%)	1.000	4 (19%)	63 (50%)	0.009
Ever smoker	7 (35%)	51 (17%)	0.060	5 (24%)	33 (26%)	0.801
HIV positive	0	3 (1%)	1.000	0	1 (1%)	1.000
Diabetes mellitus	0	10 (3%)	0.654	1 (5%)	2 (2%)	0.352
Index case clinical data						
Bilateral lung disease	4 (20%)	115 (39%)	0.080	10 (48%)	58 (46%)	0.780
Cavitary lung lesion	6 (30%)	75 (26%)	0.786	7 (33%)	27 (22%)	0.227
Smear grade >1+	8 (40%)	67 (23%)	0.168	4 (19%)	29 (23%)	0.565
QFT-Plus results at baseline (M0)						
TB1-nil IFN-γ (IU/ml)	0.14 ± 0.13	−0.02 ± 0.43	<0.001	0.11 ± 0.07	0.00 ± 0.10	<0.001
TB2-nil IFN-γ (IU/ml)	0.12 ± 0.11	−0.02 ± 0.42	<0.001	0.12 ± 0.09	−0.01 ± 0.10	<0.001
TB1-nil IFN-γ ≥0.03 IU/ml	16 (80%)	72 (24%)	<0.001	17 (81%)	26 (21%)	<0.001
TB2-nil IFN-γ ≥0.06 IU/ml	15 (75%)	59 (20%)	<0.001	17 (81%)	23 (18%)	<0.001
CD8 IFN-γ response (IU/ml)	−0.01 ± 0.07	0.00 ± 0.07	0.302	0.01 ± 0.06	−0.01 ± 0.07	0.440
M0 QFT-Plus stratification			<0.001			<0.001
TB1-nil <0.03 and TB2-nil <0.06 IU/ml	4 (20%)	212 (72%)		4 (19%)	95 (76%)	
TB1-nil ≥0.03 or TB2-nil ≥0.06 IU/ml	1 (5%)	33 (11%)		0	11 (9%)	
TB1-nil ≥0.03 and TB2-nil ≥0.06 IU/ml	15 (75%)	49 (17%)		17 (81%)	19 (15%)	

^a Among 329 initially QFT-Plus negative contacts with QFT-Plus testing at months 6, 314 contacts were included for analysis of stringent conversion after excluding 15 with indeterminate QFT-Plus results at months 6. Out of the 314 participants, there were missing data for smoking status, diabetes mellitus, radiographic findings, and sputum smear results in 21, 28, 49, and 66 subjects, respectively.

^b There were missing data for diabetes mellitus, radiographic findings, and sputum smear results in 2, 36 and 51 subjects, respectively. Continuous and categorical data are expressed as mean ± standard deviation (SD) or median with interquartile range [IQR] and number (%).

QFT-Plus indicates QuantiFERON-TB Gold Plus; TB1-nil, the difference of Interferon-gamma (IFN-γ) between tube with TB1 antigen and without stimulation (nil); TB2-nil, the difference of IFN-γ between tube with TB2 antigen and without stimulation (nil); TB2-TB1, the difference of IFN-γ between tube with TB1 antigen and tube with TB2 antigen stimulation.

Discussion

In this study, we completed serial QFT-Plus monitoring of contacts of TB patients and found that 10% of participants with QFT-Plus negative results at baseline had converted to high-positive results (e.g., stringent conversion) by 6–12 months after presumed exposure. A baseline IFN-γ response of TB1 ≥0.03 and TB2 ≥0.06 IU/ml, while still negative by the manufacturer's instructions,⁸ was highly predictive of the stringent conversion with a sensitivity of 81% and specificity of 85% and a 19.6-fold risk of conversion over 6–12 months.

Our findings are consistent with previous longitudinal studies. A cohort study in India documented a 12% annual incidence of stringent QFT-GIT conversion (IFN-γ change from <0.35 to ≥0.70 IU/ml),¹⁷ and another recent cohort study in healthy adolescents in South Africa found that 20% of QFT-GIT negative persons experienced stringent conversions (IFN-γ change from <0.2 at baseline to >0.7 IU/ml) at 12 months.¹⁸ Importantly, stringent conversions were associated with 10-fold increase in incidence of TB disease over a

2-year period,¹⁸ suggesting a very real impact of IGRA conversion on future risk of TB disease. To our knowledge, the present study is the first that uses QFT-Plus to monitor the IFN-γ response "journey" in TB contacts. The real-world clinical relevance and additional contribution of our findings suggests the global health community is missing an opportunity to treat a small, clearly identifiable, high-risk subpopulation of baseline QFT-Plus negative contacts of TB patients. Of note, baseline TB1 IFN-γ response ≥0.03 IU/ml (alone) was associated with a 14-fold increased risk of stringent conversion, suggesting a significant additive value of TB2 ≥0.06 IU/ml in the predictive model. Our findings highlight the potential synergistic role of CD4 and CD8 IFN-γ response in TB contacts undergoing active transition from LTBI to a stage at higher risk of TB disease. However, the TB1 and TB2 values indicated are very low, which may differ over different laboratories. Thus, its clinical values should be confirmed in other larger and different settings.

Our focus on the CD8 response in the QFT-Plus results also showed that while over 15% of our TB patients had strong CD8 responses (TB2-TB1 >0.6 IU/ml) in their QFT-

Table 4 Analysis for factors associated with stringent conversion (SC) in initially QFT-negative contacts.

Variables	Crude OR (95% CI)	P value	Age- & Sex-Adjusted OR (95% CI)	P value
1) M6 SC as the outcome (n = 314)				
M0 TB1-nil IFN- γ ≥ 0.03 IU/ml	12.333 (3.994–38.082)	<0.001	13.603 (4.333–42.708)	<0.001
M0 TB2-nil IFN- γ ≥ 0.06 IU/ml	11.949 (4.175–34.201)	<0.001	12.620 (4.374–36.409)	<0.001
M0 QFT-Plus stratification				
TB1-nil <0.03 and TB2-nil <0.06 IU/ml	1.0 (Reference)		1.0 (Reference)	
TB1-nil ≥ 0.03 or TB2-nil ≥ 0.06 IU/ml	1.606 (0.174–14.814)	0.676	1.716 (0.185–15.932)	0.635
TB1-nil ≥ 0.03 and TB2-nil ≥ 0.06 IU/ml	16.224 (5.159–51.028)	<0.001	17.601 (5.520–56.121)	<0.001
2) M12 SC as the outcome (n = 147)				
M0 TB1-nil IFN- γ ≥ 0.03 IU/ml	16.183 (5.014–52.229)	<0.001	19.148 (5.594–65.549)	<0.001
M0 TB2-nil IFN- γ ≥ 0.06 IU/ml	18.848 (5.795–61.302)	<0.001	20.126 (5.920–68.414)	<0.001
M0 QFT-Plus stratification				
TB1-nil <0.03 and TB2-nil <0.06 IU/ml	1.0 (Reference)		1.0 (Reference)	
TB1-nil ≥ 0.03 or TB2-nil ≥ 0.06 IU/ml	—		—	
TB1-nil ≥ 0.03 and TB2-nil ≥ 0.06 IU/ml	21.250 (6.430–70.228)	<0.001	23.642 (6.752–82.782)	<0.001
3) M6 or M12 developing SC (n = 423)				
M0 TB1-nil IFN- γ ≥ 0.03 IU/ml	13.580 (6.053–30.466)	<0.001	14.389 (6.345–32.627)	<0.001
M0 TB2-nil IFN- γ ≥ 0.06 IU/ml	14.799 (6.771–32.342)	<0.001	16.063 (7.265–35.518)	<0.001
M0 QFT-Plus stratification				
TB1-nil <0.03 and TB2-nil <0.06 IU/ml	1.0 (Reference)		1.0 (Reference)	
TB1-nil ≥ 0.03 or TB2-nil ≥ 0.06 IU/ml	0.901 (0.110–7.397)	0.922	0.956 (0.116–7.889)	0.967
TB1-nil ≥ 0.03 and TB2-nil ≥ 0.06 IU/ml	18.129 (7.968–41.249)	<0.001	19.565 (8.484–45.116)	<0.001

TB1-nil ≥ 0.03 and TB2-nil ≥ 0.06

TB1-nil, the difference of Interferon-gamma (IFN- γ) between tube with TB1 antigen and without stimulation (nil); TB2-nil, the difference of IFN- γ between tube with TB2 antigen and without stimulation.

Plus results, 9% of the contacts diagnosed as LTBI did as well, which is in line with other reports.⁹ Interestingly, among participants with positive QFT-Plus results, strong CD8 response yielded a 2.7-fold odds of differentiating TB disease from LTBI, suggesting a moderately added value of the TB2 tube since our data showed that TB1 response itself was not different between the two subgroups. Although CD8 IFN- γ response at baseline, which should be very low by definition in QFT-Plus negative condition, was not predictive of QFT-Plus stringent conversion, other prognostic values of CD8 IFN- γ response deserves further investigation in various clinical settings.

Moreover, our study found that bilateral lung disease of index TB patients was not only marginally correlated in strong CD8 response in TB patients but also significantly associated with strong CD8 response in contacts. Our findings suggest that lung inflammatory status of index TB patients was associated with CD8 IFN- γ response in their contacts. A clinical study revealed that a modern MTB lineage was associated with severe lung damage and increased level of TNF- α but not related to smear status of TB patients.²⁰ Another contact investigation study reported that certain MTB strains in TB patients may induce different IFN- γ response in their contacts.¹² Thus, it is possible that infection caused by certain virulent MTB lineages may result in more severe lung inflammation leading to bilateral lung disease in TB patients and induce strong CD8 responses in TB patients as well as elevated CD8 responses in their contacts. Our study joins this growing body of literature, but this hypothesis warrants further MTB lineage infection modeling to support possible associations.

We also found that smear status of index TB patients was associated with higher TB1 and TB2 IFN- γ levels, but not CD8 responses, in their contacts. Two recent mouse models have reported that CD4⁺ T cells is more important than CD8⁺ T cells to control the bacterial load in the early phase of MTB infection,²¹ and that in immune deficient mice infected by *Mycobacterium bovis* Bacillus Calmette-Guérin, CD4⁺ T cells transferring to mice reduced bacterial burden in the lung but CD8⁺ T cells transferring did not.²² Thus, we hypothesize that CD4 immune response may be more sensitive to high bacterial burden upon MTB exposure, which may in turn result in correspondingly high CD4 generated TB1 and TB2 IFN- γ response in contacts. Our findings further suggest smear status of index case may be preferentially associated with CD4 IFN- γ response in contacts.

The present study has some limitations. First, due to the COVID-19 pandemic, some of the missing data was not recoverable and identification of incident TB in contacts on a regular basis was not always possible. There is a need for additional study to investigate the association between QFT-Plus response at baseline and incident TB during follow-up in contacts with untreated LTBI. Second, we were unable to confirm if contacts had community exposure to other TB patients during follow-up, and this is especially relevant given the study location known ongoing local TB transmission. Third, we did not analyze MTB lineage isolated from the index case, nor the environment of the household and exposure period between index TB patients and contacts.^{6,10,12} Finally, the impact of HIV on QFT-Plus conversion could not be assessed because there was few cases of HIV in the cohort.³

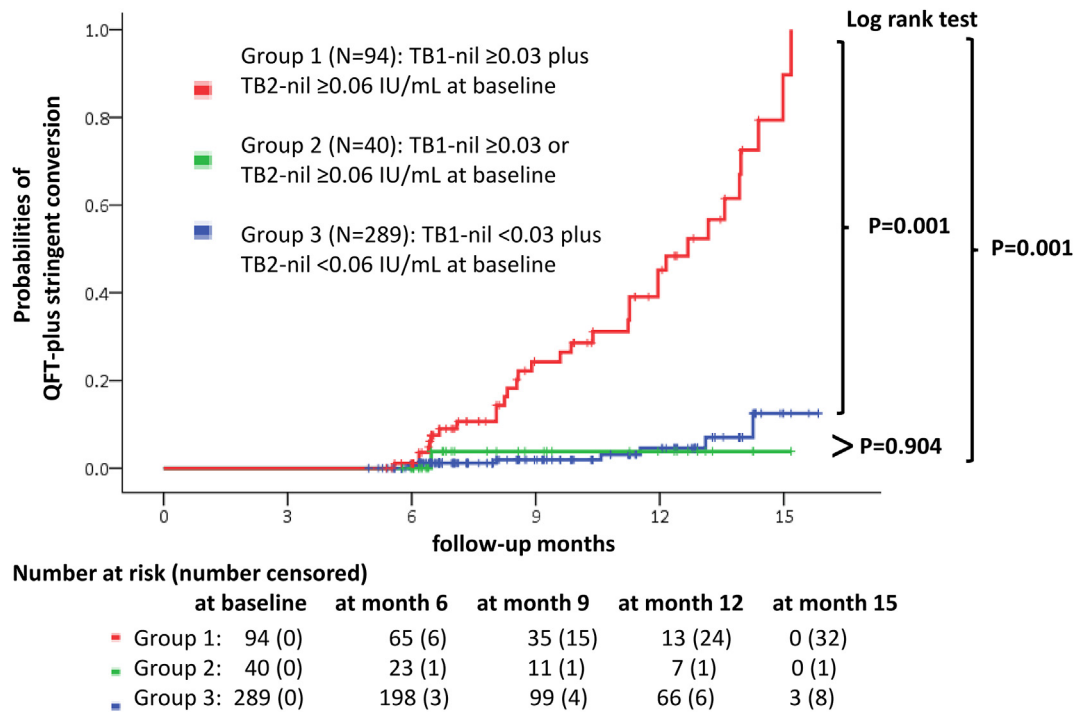


Figure 4. Kaplan–Meier survival curve showing the probability of QFT-Plus stringent conversion in contacts stratified by baseline TB1 and TB2 interferon gamma responses ($n = 423$).

In conclusion, the combined baseline TB1 and TB2 IFN- γ response was highly predictive of a QFT-Plus stringent conversion during the 6–12 months of follow-up in TB contacts. Moreover, increased CD4 and CD8 responses in contacts were associated with smear status and bilateral lung disease of index TB patients, respectively. Furthermore, among positive QFT-Plus population, the CD8 IFN- γ response, but not TB1 or TB2 response, was higher in TB patients compared with contacts with LTBI, suggesting additional diagnostic value of CD8 response. Further studies are warranted to assess the potential impact of setting thresholds for close monitoring based on baseline CD4 and CD8 IFN- γ responses from the QFT-Plus assay among contacts.

Funding

The authors thank the US National Institutes of Health (Grant Number: R01AI137681) and the US Department of Defense (Award Number W81XWH-18-1-0253) for their supports in conducting this study. Research reported in this publication was supported by the National Institute Of Allergy And Infectious Diseases of the National Institutes of Health under Award Number R01AI137681. The content is solely the responsibility of the authors and does not necessarily represent the official views of the National Institutes of Health. This work was supported by the U.S. Army Medical Research and Development Command, through the Peer Reviewed Medical Research Program under Award No. W81XWH1810253. Opinions, interpretations, conclusions and recommendations are those of the author and are not necessarily endorsed by the U.S.

Army. The authors also thank the National Science and Technology Council, Taiwan (MOST 111-2314-B-075 -059 and NSTC 112-2314-B-075 -059) for supporting the publication of this study.

Acknowledgements

The authors would like to thank staffs working at the Republic of Moldova's National TB Program (NTP), the partnership TB clinics, contact tracing program, and the National TB Reference Laboratory (NTRL) for providing assistance in this study. The authors thank Taipei Veterans General Hospital, Taiwan, for providing a scholarship for Dr. Pan SW to study and conduct research with Prof. Rodwell TC at the University of California San Diego.

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Appendix A. Supplementary data

Supplementary data to this article can be found online at <https://doi.org/10.1016/j.jmii.2023.07.014>.

Identification of differentially recognized T cell epitopes in the spectrum of tuberculosis infection

Received: 13 April 2023

Accepted: 12 January 2024

Published online: 26 January 2024

 Check for updates

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There is still incomplete knowledge of which *Mycobacterium tuberculosis* (*Mtb*) antigens can trigger distinct T cell responses at different stages of infection. Here, a proteome-wide screen of 20,610 *Mtb*-derived peptides in 21 patients mid-treatment for active tuberculosis (ATB) reveals IFN γ -specific T cell responses against 137 unique epitopes. Of these, 16% are recognized by two or more participants and predominantly derived from cell wall and cell processes antigens. There is differential recognition of antigens, including TB vaccine candidate antigens, between ATB participants and interferon-gamma release assay (IGRA +/–) individuals. We developed an ATB-specific peptide pool (ATB116) consisting of epitopes exclusively recognized by ATB participants. This pool can distinguish patients with pulmonary ATB from IGRA +/– individuals from various geographical locations, with a sensitivity of over 60% and a specificity exceeding 80%. This proteome-wide screen of T cell reactivity identified infection stage-specific epitopes and antigens for potential use in diagnostics and measuring *Mtb*-specific immune responses.

Tuberculosis is the ninth leading cause of death worldwide and the leading cause from a single infectious agent, ranking above HIV/AIDS. The World Health Organization (WHO) estimates that approximately one-quarter of the world's population (1.7 billion total) is infected with *Mycobacterium tuberculosis* (*Mtb*). In 2021 *Mtb* was responsible for 1.6 million deaths and ~10 million new infections¹

Infection with *Mtb* manifests as a spectrum of diseases ranging from asymptomatic subclinical infection (latent infection, LTBI) to active disease (ATB)^{2,3}. To date, tuberculin skin test (TST) and interferon-gamma release assays (IGRA), which detect an

immunological response against *Mtb*, are diagnostic tests for latent infection^{4,5}. Importantly, the classically used tuberculin skin test can be positive in both LTBI and BCG vaccinated participants and thus cannot distinguish them^{6,7}. A major advance in the diagnosis of *Mtb* infection was represented by the introduction of IGRA tests that consist of ex vivo analysis of peripheral blood cells for a cytokine (IFN γ) response to peptide pools spanning the ESAT-6 and CFP10 antigens recognized by T cells⁸. As these antigens are absent from *M. bovis* BCG and most nontuberculous mycobacteria (NTMs), such responses can distinguish prior or current *Mtb* infection from BCG vaccination and NTM

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diseases. However, these tests cannot reliably discriminate between active TB and LTBI⁹. These observations indicate the need for a more extensive search for a panel of antigens that can distinguish LTBI and active disease.

T cells are critical in the host immune response against *Mtb* infection. The responses against *Mtb* infection involve classically restricted CD4 and CD8 $\alpha\beta$ T cells^{10,11} and non-classically restricted T cells such as NKT (CD1), MAIT (MRI), and $\gamma\delta$ T cells^{12–14}. Among these, CD4 T cells represent a major component of T cell response against *Mtb* infection¹⁵. Individuals with low levels of CD4 T cells, such as those who are HIV positive, are more vulnerable to both primary and reactivation of TB¹⁶. Multiple studies have revealed the recognition of T-cell epitopes from *Mtb* among diverse populations, including individuals with TB disease, LTBI, BCG vaccination, or exposure to *Mtb* and/or NTM^{17–20}, also reviewed in ref. 21. T cell responses at the antigen and epitope levels are highly complex, particularly in complex organisms. They can involve multiple antigens and hundreds of epitopes^{22–25}, with broader patterns of immunodominance observed in humans compared to genetically homogeneous murine model^{26–28}. While the mechanisms underlying T cell immunodominance and breadth have been extensively studied in murine models and, to some extent, in humans^{22,29–32}, a quantitative assessment of the complexity of responses during natural infections at the population level is currently lacking. Most immuno-profiling studies have focused on individual antigens or a limited set of epitopes, assuming they represent the entire pathogen-specific response^{33,34}. It remains uncertain to what extent underestimating the true complexity of these responses could affect the outcomes of immuno-profiling studies. Therefore, a comprehensive understanding of *Mtb* epitopes across TB disease states is crucial. *Mtb* expresses different proteins during various stages of infection, resulting in stage-specific immune responses. Thus, identifying novel CD4 T cell epitopes at different stages of TB could aid in discovering new diagnostic and vaccine candidates against TB. Previously, our team conducted a proteome-wide screen to detect HLA class II restricted epitopes and antigens recognized in healthy IGRA+ participants without any signs of active TB²². This screen included 4000 ORFs of the *Mtb* genome and approximately 20,000 predicted HLA class II epitopes. T cell reactivity against these epitopes was measured using ELISPOT to detect IFN γ response ex vivo. It is equally important to screen these epitopes in ATB disease. Identifying the ATB-specific antigens will be important as boosting immune responses against antigens expressed during the active phase of infection might help translate into reduced incidence or reactivation of infection.

This study explored the possibility that distinct *Mtb* disease states (i.e., healthy IGRA+ participants and patients with active pulmonary TB) respond to differential *Mtb*-derived epitopes and/or elicit different *Mtb*-specific T cell responses. We hypothesized that this differential reactivity could be attributed to variations in response magnitude, specificity (i.e., which antigens are recognized), breadth (i.e., how diverse the response is), and the phenotype of the specific T cells. By conducting a comprehensive proteome-wide screening for *Mtb*-derived T cell epitopes, we identified and characterized epitopes specifically recognized by participants with active pulmonary TB, suggesting their potential utility as diagnostic markers and vaccination candidates.

Results

Broad and heterogeneous T cell responses against *Mtb*-derived HLA class II predicted epitopes in participants with ATB

Detailed knowledge regarding the antigens recognized in TB disease and associated phenotypes is relevant for understanding TB immunopathology and for vaccine and diagnostic applications. We previously used a proteome-wide peptide library of 20,610 *Mtb*-derived 15-mer peptides predicted promiscuous HLA class II binders to define HLA class II restricted epitopes and associated antigens in

healthy IGRA+ participants (i.e., LTBI) from San Diego, US²². Here, we used the same comprehensive library to determine the repertoire of T cell antigens and epitopes recognized in ATB.

The proteome-wide peptide library was arranged into 1036 peptide pools of 20 peptides each. T cell reactivity against the 1036 peptide pools was measured by ex vivo production of IFN γ and IL-17 using Fluorospot assays and PBMCs from 21 participants mid-treatment for their ATB infection (3–4 months post diagnosis) recruited from the Universidad Peruana Cayetano Heredia site (Peru). This mid-treatment timepoint was selected based on the participants being eligible for a leukapheresis donation, thus allowing the entire peptide library to be tested in each participant. IFN γ was selected based on the important role of this cytokine in immune responses against *Mtb*^{35–38}, similarly, IL-17 plays a role in TB pathogenesis³⁹.

No IL-17 was detected in response to the peptide pool stimulations. Therefore, we selected peptide pools based on IFN γ response for deconvolution to identify individual epitopes. A total of 78 unique peptide pools were selected for deconvolution, corresponding to the ten peptide pools with the highest response magnitude per participant and/or that were recognized by at least two different participants. A total of 137 individual epitopes were identified (Supplementary Data 1). Each participant recognized an average of 8 unique epitopes (range 1–27, median 7), underlining the breadth of responses to *Mtb* (Fig. 1A). Among the 137 individual epitopes, 22 (16%) were recognized by multiple participants (Fig. 1B). When epitopes were ranked based on magnitude, the top 55 epitopes accounted for 80% of the total response (Fig. 1C). In conclusion, the breadth of responses detected in ATB is broad, although narrower ($p = 0.002$, two-tailed unpaired Student's t-test) compared to the previous results in healthy IGRA+ participants who recognized, on average, 24 epitopes²². These identified epitopes from the proteome-wide screen in participants with ATB have been submitted to IEDB (Immune Epitope Database) and can be found under the submission ID: 1000914.

Distinct immunodominant antigens predominantly from cell wall and cell processes in ATB participants

The epitopes were mapped to individual *Mtb* ORFs using H37Rv as a reference genome. A total of 97 ORFs were recognized, with each participant recognizing, on average, 6 ORFs (median 5, Fig. 2A). As expected, the well-known antigens Rv0288 (TB10.4), Rv3875 (ESAT-6), Rv3874 (CFP10), and Rv3615c (included in the “ESAT-6 free” IGRA test⁴⁰) were the most frequently recognized. However, nine novel antigens, which have not been previously described as antigens for *Mtb*, were also identified (Supplementary Data 1).

Using the Mycobrowser tool⁴¹, we determined the protein categories to which the identified antigenic ORFs belonged. As previously observed for IGRA+ participants⁴², essentially every protein category was represented (Fig. 2B). However, the ORFs antigenic in ATB were predominantly found in the cell wall and cell processes categories, followed by conserved hypotheticals, PE/PPE, and intermediary metabolism and respiration categories (Fig. 2B). Compared to the H37Rv genome, the antigenic ORFs were overrepresented in the cell wall, cell processes category, and the PE/PPE categories and underrepresented in conserved hypotheticals (Fig. 2C). The overrepresentation of the cell wall and cell processes category appears to be specific for the ATB cohort because these antigen categories were not overrepresented in the previous screen of IGRA+ participants²².

Reactivity in healthy IGRA+ participants²² previously identified three “antigenic islands” of clustered antigen genes that comprised secreted and non-secreted *Mtb* proteins involved in type 7 secretion systems. Indeed, all three antigenic islands were also identified in the present screen of ATB participants. (Fig. 2D). In contrast to IGRA+ participants in the previous screen, the breadth of responses to antigens targeting the antigenic islands was narrower in the participants with ATB (Fig. 2E). For example, for island 1, only 3 antigens

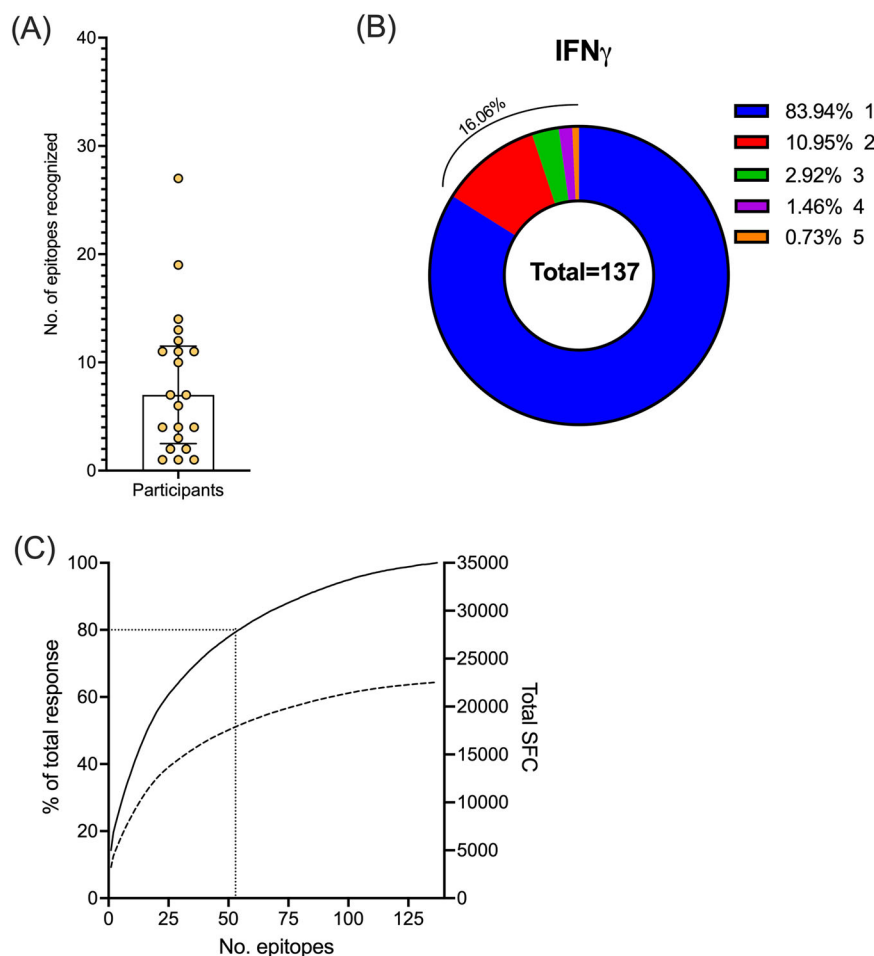


Fig. 1 | Breadth and dominance of epitopes in mid-treatment ATB participants. **A** Number of epitopes recognized by each participant. Each dot is one participant, $n = 21$; median $\pm$ interquartile range is shown. **B** Distribution of recognized epitopes by the number of participants recognizing each epitope. **C** Epitopes ranked based

on the magnitude of response (solid line - % of total response, dotted line - total spot forming cells (SFC)). Black dotted lines indicate the top 55 epitopes. Source data are provided as a Source data file.

represented in the predicted peptide library of 20,610 peptides were recognized by participants with ATB, compared to 9 recognized by healthy IGRA+ participants. The same is true for antigens in islands 2 and 3. In conclusion, the data suggest that different patterns of antigenic ORFs might be associated with ATB vs. healthy IGRA+ participants.

Hierarchy in reactivity against TB Vaccine and IGRA antigens

Previous studies showed that the proteome-wide library of predicted promiscuous HLA class II binders captures about 50% of the total reactivity^{22,43}. To further evaluate T cell responses against TB vaccine candidate and IGRA antigens (see methods), we tested 517 15-mer peptides overlapping by 10 amino acids spanning these antigens. Positive pools were deconvoluted to identify individual T cell epitopes. Overall, 67% of the ATB participants recognized epitopes from at least one antigen; on average, these participants recognized 2 different antigens (range 1–4). This is similar to our previous reports highlighting the inter-individual variability of epitope-specific responses⁴².

We next compared the magnitude and frequency of the response for these antigens in the ATB participants with what was previously observed in healthy IGRA+ participants from South Africa⁴². The most frequently recognized antigens in ATB and IGRA+ alike were Rv0288 (TB10.4), Rv3875 (ESAT-6), Rv3874 (CFP10), and Rv1196 (PPE18) (Fig. 3A, B). However, some antigens were differentially recognized in the two cohorts. Specifically, Rv3875 was more frequently recognized than Rv3874 in ATB vs. IGRA+ participants. The Rv1813c antigen was

reactive in participants with ATB and not in IGRA+. Finally, the Rv3619c (EsxV), Rv2660c, Rv0125 (Mtb32a), and Rv2608 (PPE42) antigens were reactive in IGRA+ and completely unreactive in participants with ATB (Fig. 3A, B).

The proteome-wide screen detected the two most reactive vaccine antigens, Rv0288 and Rv3874. Rv3875 was not detected in the proteome-wide screen, likely due to its small size, with only 2 peptides representing it in the proteome-wide library. The results confirm that the antigens detected in the proteome-wide screen are the most frequently recognized together with Rv3875 and that the other vaccine antigens account for a small fraction of the response in this ATB cohort. In conclusion, the screen of vaccine and IGRA antigens, together with the proteome-wide screen, identified a total of 174 epitopes, which were next investigated for functionality and differential reactivity in further experiments.

Differential patterns of cytokine expression and memory subsets in CD4 T cell responses against different protein antigen categories

The responses associated with the 174 identified epitopes were characterized in more detail in a randomly selected subset of the participants with ATB (mid-treatment) from Peru by intracellular cytokine secretion assays to characterize T cell responses specific to antigens from the different protein categories. One epitope pool (PC85) corresponded to epitopes from antigens in the cell wall and cell processes category, and a separate pool encompassed epitopes from other

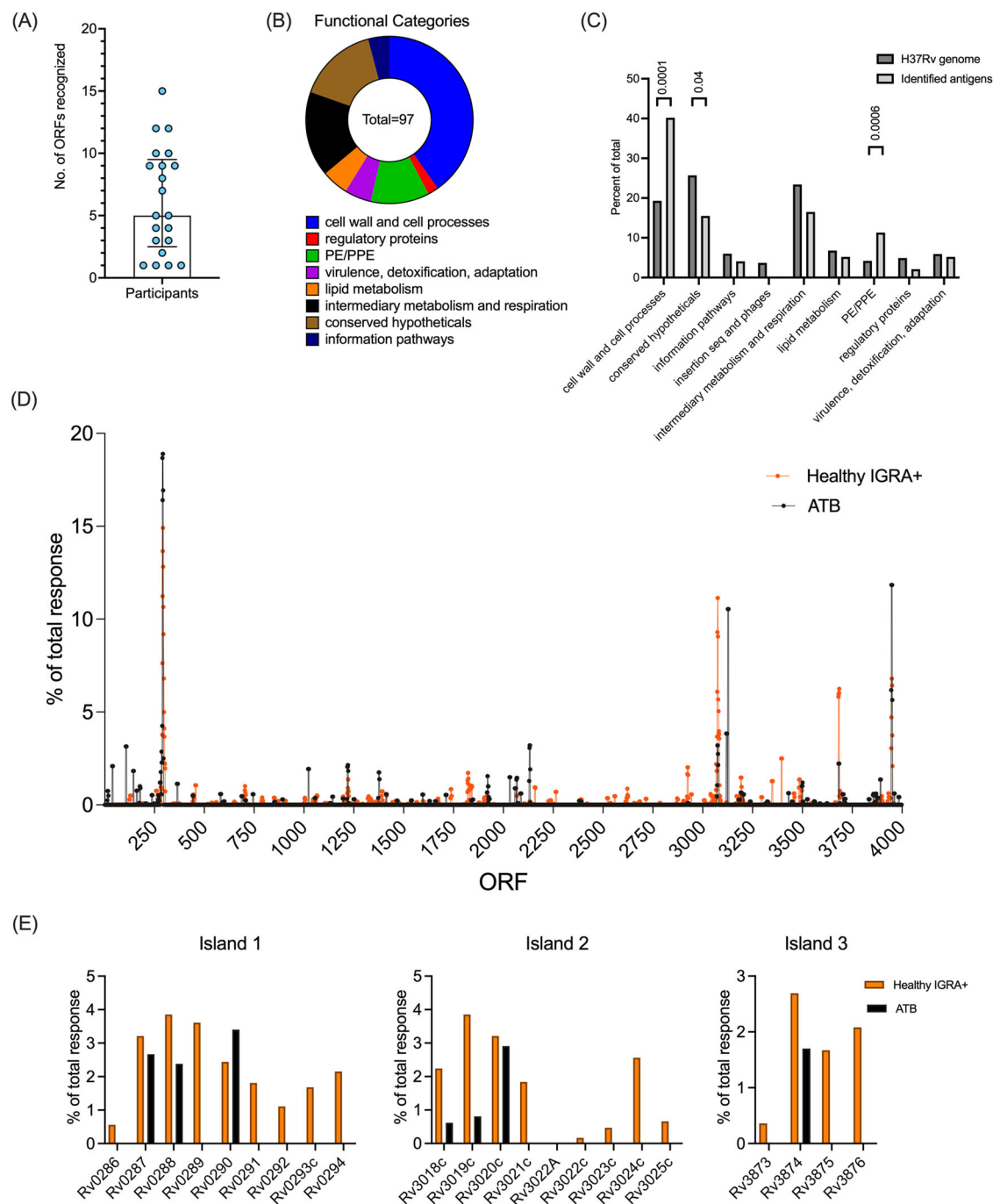


Fig. 2 | Immunodominant antigens in ATB. A Number of open reading frames (ORFs) corresponding to recognized epitopes by each participant. Each dot represents one participant, $n = 21$, median $\pm$ interquartile range is shown. **B** Distribution of recognized ORFs per protein category. **C** The identified antigens (black bars) were divided into protein categories (Mycobrowser) and compared to

the H37Rv genome (grey bars). Chi-square test (two-sided). **D** Antigenic islands identified by a 5-gene window spanning the H37Rv genome. ATB (black) compared to healthy IGRA+ (orange²²). **E** Proteins within each antigenic island, % of total response per antigen across the proteome-wide screen. ATB (black bars) and healthy IGRA+ (orange bars). Source data are provided as a Source data file.

protein categories (PC71) (Supplementary Data 1). As a comparator, we included the previously described MTB300 pool, based on epitopes recognized in IGRA+ participants²², which includes peptides from all functional categories.

As expected, based on the peptide library design to bind HLA class II alleles, most of the response was mediated by CD4 T cells (Fig. 4A). Among them, the frequency of TNF α + CD4 cells was relatively higher than IFN γ ($p = 0.0006$ for PC71) or IL-2 ($p = 0.04$ for PC85). In addition, some responses were detected in CD8 + T cells (Fig. 4B), likely due to nested HLA class I binding epitopes within the 15-mer peptides.

Frequencies of cytokine-expressing CD4 T cells in response to both pools were similar to MTB300 (IFN γ , $p = 0.98$ for PC85 and PC71; IL-2, $p = 0.31$ and 0.97 for PC85 and PC71 respectively; TNF α , $p = 0.78$ and 0.51 for PC85 and PC71 respectively) (Fig. 4A, gating strategy shown in Figure S1). The vast majority of cytokine producing CD4 T cells expressed a single cytokine TNF α or IFN γ , followed by TNF α + IL-2 + and TNF α + IFN γ + dual cytokine expressed cells with none to very few IFN γ + IL-2 + cells and triple cytokine producing cells (Fig. 4C, D). A higher frequency of single TNF α -producing T cells was present compared to other single (IFN γ $p = 0.01$ (PC71), IL-2 $p = 0.01$ (PC85)),

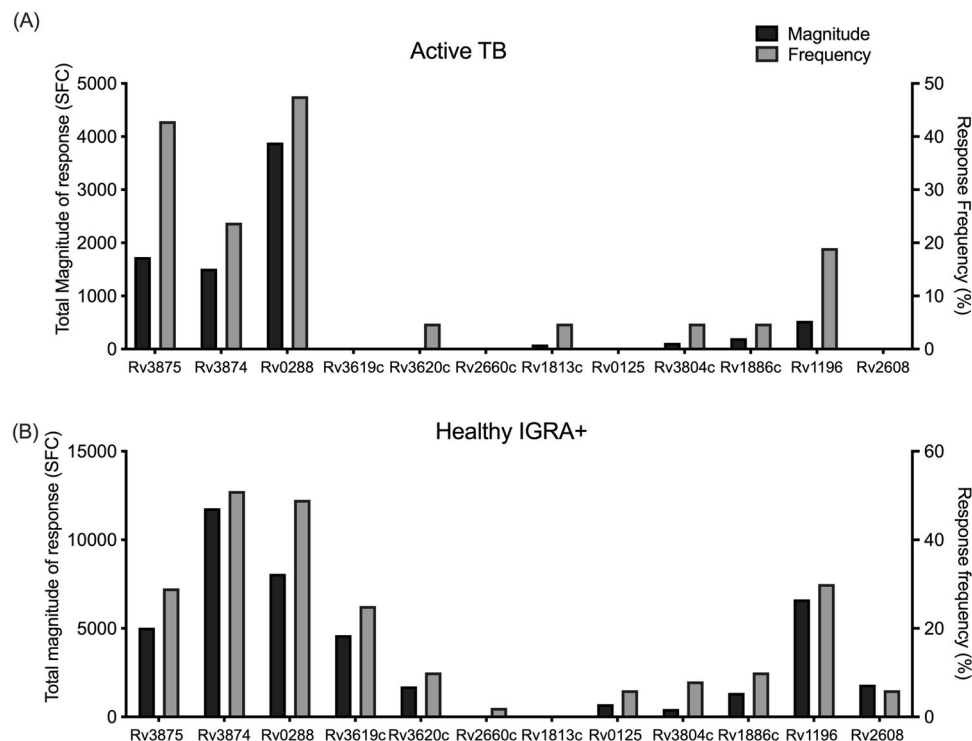


Fig. 3 | Hierarchy in T cell reactivity against TB vaccine and IGRA antigens. Magnitude of response, expressed as the total magnitude of response (black bars, left y-axis) or frequency of participants responding (grey bars, right y-axis),

amongst the participants. **A** ATB, $n = 21$. **B** Healthy IGRA+, $n = 63^{42}$, for comparison purposes. Rv number for each antigen are indicated on the x-axis. Source data are provided as a Source data file.

dual (IFN γ + IL-2+ $p = 0.0006$ (PC71), $p = 0.0002$ (PC85), TNF α + IFN γ + $p = 0.002$ (PC71), $p = 0.01$ (PC85), TNF α + IL-2 + $p = 0.02$ (PC71), $p = 0.008$ (PC85)), or triple ($p = 0.0008$ (PC71), $p = 0.0003$ (PC85) cytokine producing cells (Fig. 4C).

Next, we characterized which T cell memory subset was responsible for the reactivity. Memory subset phenotypes were determined using antibodies to CD45RA and CCR7. The majority of the cytokine producing CD4 T cells were effector memory (CD45RA-CCR7-), and as expected few were naïve (CD45RA + CCR7 +) cells (Fig. 4E, F). For IFN γ there was no difference between the CD4 memory subsets comparing the different peptide pools (Fig. 4E). For IL-2 there was a significantly higher frequency of effector memory T cells ($p = 0.0007$ and 0.03) and a lower frequency of T_{EMRA} (CD45RA + CCR7 +) T cells ($p = 0.001$ and 0.02) in response to PC85 compared to the PC71 and MTB300 respectively (Fig. 4F). The PC85 stimulation also resulted in a significantly higher frequency of effector memory TNF α -producing cells ($p = 0.0003$ and 0.008) and a lower frequency of central memory cells ($p = 0.04$ and 0.02) than PC71 and MTB300, respectively (Fig. 4G). This finding suggests distinct differentiation of the CD4 memory subsets in response to epitope pools representing different functional categories.

An ATB-specific T cell epitope pool that can distinguish ATB from healthy IGRA+ participants

The data for both the proteome-wide screen and the TB vaccine and IGRA antigens suggests that certain antigens and epitopes are differentially recognized in ATB vs. healthy IGRA+ individuals. Accordingly, we next tested whether reactivity to these “ATB-specific epitopes,” could differentiate individuals with ATB at diagnosis, as a cohort more relevant for diagnostic purposes vs. IGRA+ and IGRA- healthy controls. Peptides that were exclusively recognized by participants with ATB (mid-treatment) as compared to our previous studies in IGRA+ participants were pooled into an ATB-pool (ATB116) consisting of 116 peptides (Supplementary Data 1). Reactivity to

MTB300 (constructed based on reactivity in IGRA+ participants) was utilized as a comparator, and none of the peptides in MTB300 overlap with ATB116.

IFN γ response was determined in a Sri Lankan cohort, including 24 patients with ATB (recruited at diagnosis), 25 IGRA+, and 43 IGRA- participants. ATB116 stimulation resulted in significantly higher IFN γ in patients with ATB compared to both IGRA+ and IGRA- controls (Fig. 5A), with 63% of ATB responding compared to 8% of IGRA+ and 16% of IGRA- participants. The frequency of participants responding to MTB300 was similar between the cohorts. MTB300 could not discriminate between ATB and IGRA+, but showed a significantly higher magnitude of reactivity in IGRA+ compared to IGRA- (Fig. 5A). MTB300 contains epitopes that are conserved in nontuberculous mycobacteria, which have been shown to correlate with reactivity observed in IGRA- participants⁴⁴. Similar results were obtained when responses were measured by ICS rather than Fluorospot (Fig. 5B). The IFN γ , IL-2, and TNF α cytokine production was determined in 9 participants with ATB (at diagnosis) and 9 IGRA- participants from Sri Lanka. The frequency of IFN γ and TNF α -producing CD4 cells against the ATB116 was significantly higher in ATB compared to IGRA- participants (Fig. 5B). No differences were observed between these cohorts in response to MTB300.

These results were independently confirmed in an independent cohort from the Republic of Moldova of ATB (at diagnosis and mid-treatment), IGRA+, and IGRA- participants (household contacts of an ATB index case). Similar to what was observed in the case of the Sri Lankan cohort, ATB116 stimulation resulted in a significantly higher magnitude of response in patients with ATB, both at diagnosis and mid-treatment (100% responders), compared to IGRA+ and IGRA- controls (13.4% vs. 15.3% responders; Fig. 5C). MTB300 also showed higher magnitude of response in ATB, both at diagnosis and mid-treatment (100% responders) compared to IGRA+ and IGRA- controls (68% vs. 34.6% responders (Fig. 5C).

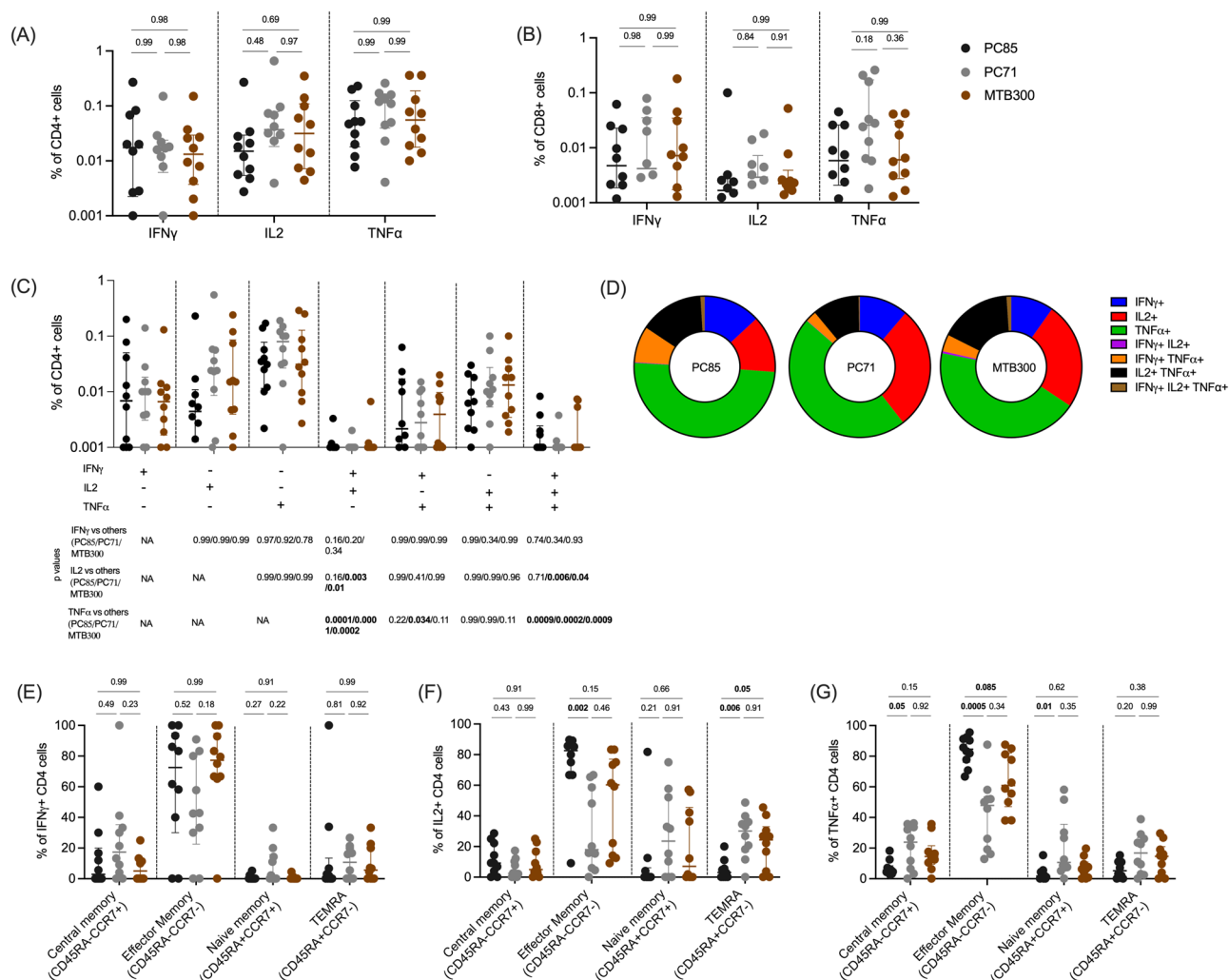


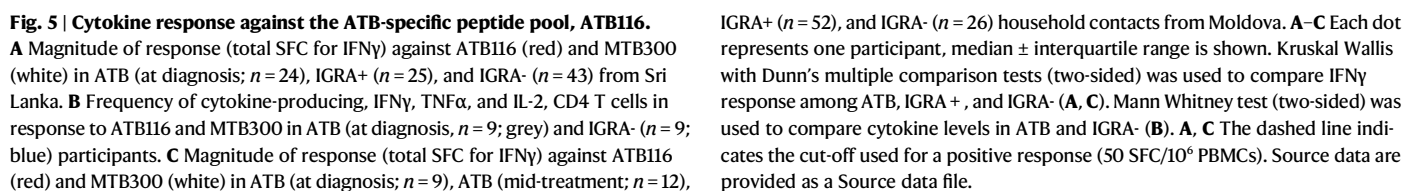
Fig. 4 | T cell responses specific for different protein categories. **A** Frequency of cytokine-producing, IFN γ , TNF α , and IL-2, CD4 T cells in response to PC85, PC71, and MTB300. **B** Frequency of cytokine-producing, IFN γ , TNF α , and IL-2, CD8+ T cells in response to PC85, PC71, and MTB300. **C** Percentage pool-specific IFN γ , TNF α , and IL-2 production by CD4 T cells expressing each of the seven possible combinations. **D** Pie charts representing single, dual and triple cytokine producing CD4 T cells. Each section of the pie chart represents a specific combination

of cytokines, as indicated by the color. **E–G** Proportion of CCR7+ CD45RA- (central memory), CCR7+ CD45RA+ (effector memory), CCR7- CD45RA+ (naïve), and CCR7- CD45RA+ (TEMRA) T cells for each peptide pool. **E** IFN γ , **(F)** IL-2, **(G)** TNF α . **(A–C, E–G)** Each point represents one participant from the Peruvian cohort (mid-treatment), $n = 10$, median $\pm$ interquartile range is shown. Kruskal Wallis with Dunn's multiple comparison test (two-sided) was used for comparison. Source data are provided as a Source data file.

Next, we calculated the sensitivity and specificity for ATB116 to explore its diagnostic potential. The sensitivity was calculated as the percentage of the participants with ATB responding to ATB116 out of the total ATB cohort. The specificity was calculated as the percentage of IGRA+/- who did not respond to ATB116 out of the total IGRA+/- participants. ATB116 demonstrated a high sensitivity of 62.5% and specificity of over 80% in distinguishing patients with ATB from those who were IGRA+ and IGRA- in the Sri Lankan cohort. Similarly, the sensitivity was 100% and the specificity was over 80% in the Moldovan cohort differentiating ATB from IGRA+ and IGRA- participants. (Table 1). The diagnostic potential by ROC curve analysis also demonstrated good predictive performance for ATB116 in distinguishing ATB from both IGRA+ and IGRA- in both cohorts (Fig. 6). In the Sri Lanka cohort, the area under the curve (AUC) was 0.83 (Fig. 6A) for distinguishing ATB from IGRA+ individuals and 0.77 (Fig. 6B) for distinguishing ATB from IGRA- individuals. Similarly, in the Moldovan cohort, the AUC was 0.93 (Fig. 6C) for distinguishing ATB from IGRA+ individuals and 0.91 (Fig. 6D) for distinguishing ATB from IGRA- individuals. These findings highlight the diagnostic potential of ATB116 as a useful tool in identifying cases of active tuberculosis.

The ATB-specific T cell response changes during treatment for ATB

Finally, to investigate the changes in T cell response during treatment, we followed 7 participants in Sri Lanka from diagnosis to mid-treatment and end of treatment. We analyzed the proportion of cytokine-producing CD4 T cells after pool stimulation. In this longitudinal cohort, we observed a significant decrease in ATB116-specific IFN γ responses between visit 1 (at diagnosis) and visit 2 (mid-treatment) (Fig. 6A). At visit 3 (end of treatment), about half the cohort had a further decrease in their response, whereas in the other half, the response against ATB116 increased again. The response against MTB300 did not change between visits 1 and 2 but was significantly decreased compared to visits 2 and 3 (Fig. 7A). The ATB116-specific IL-2 response increased between visits 1 and 3 (Fig. 7B). The response for MTB300 remained the same. Finally, the ATB116-specific TNF α response increased at visit 2 compared to both visits 1 and 3 (Fig. 7C). Again, there was no difference in the response against MTB300. These results indicate that the response against ATB116 changes during treatment for ATB and is differentially affected depending on the specific cytokine measured.



Discussion

Here, we report the first proteome-wide identification of *Mtb*-derived T cell epitopes in a cohort of patients with ATB. We have defined the epitopes from a library of over 21,000 peptides, with an in-depth investigation of the antigens in TB vaccine candidates. The lack of understanding of the broad range of *Mtb* antigens that may elicit a differential T cell response between individuals with various stages of *Mtb* infection is a bottleneck in developing diagnostic assays and vaccines. Many studies have focused on the identification of novel antigens which can be utilized for better diagnostic assays and vaccine development^{21,45–48}. Despite many efforts, only few antigens, <20, of the over 4000 ORFs in the *Mtb* genome are included in subunit vaccine candidates^{49–53}.

Comparing the results described here with those from our previous studies in healthy IGRA+ participants²² revealed differentially

recognized epitopes and antigens between ATB and IGRA+ participants. This differential recognition of antigens can be explained by *Mtb*'s expression of infection stage-specific antigens, as previously reported⁵⁴. For instance, during *Mtb* infection, *Mtb* alters its metabolic state from active replication to slow or nonreplicating, accompanied by changes in the gene expression profile and thus protein expression and antigens available to the immune system⁵⁵. The differential expression and availability of antigens at distinct stages of TB infection might exhibit distinct patterns of differentiation and restricted capacities to mediate protective immunity⁵⁶.

The proteome-wide screen in IGRA+ participants revealed three antigenic islands that were immunodominant and related to type 7 secretion systems, with secreted and secretion apparatus proteins being recognized as antigens²². Here, we found reactivity against antigens that are part of the antigenic islands, albeit with a more restricted recognition of primarily secreted proteins. The underlying cause of this is unclear, but it could be due to *Mtb*'s differential expression of proteins in different stages of TB infection. Our studies also highlighted a hierarchy of responses for the vaccine candidate and IGRA antigens in the two cohorts, with the well-studied antigens, Rv0288, Rv3875, Rv3874, and Rv1196, being the most frequently recognized. This highlights the complexity of *Mtb*-specific T cell responses and suggests the need for vaccine strategies targeting many different antigens.

Table 1 | Diagnostic potential of ATB116 distinguishing patients with ATB from IGRA+ and IGRA- participants

	Cohort	Sensitivity (95% CI)	Specificity (95% CI)
Sri Lanka	ATB vs IGRA+	62.5 (42.7–78.8)	92 (75.0–98.6)
	ATB vs IGRA-		83.7 (70.0–91.8)
Moldova	ATB vs IGRA+	100 (84.5–100)	84.6 (72.5–92.0)
	ATB vs IGRA-		80.9 (66.5–93.9)

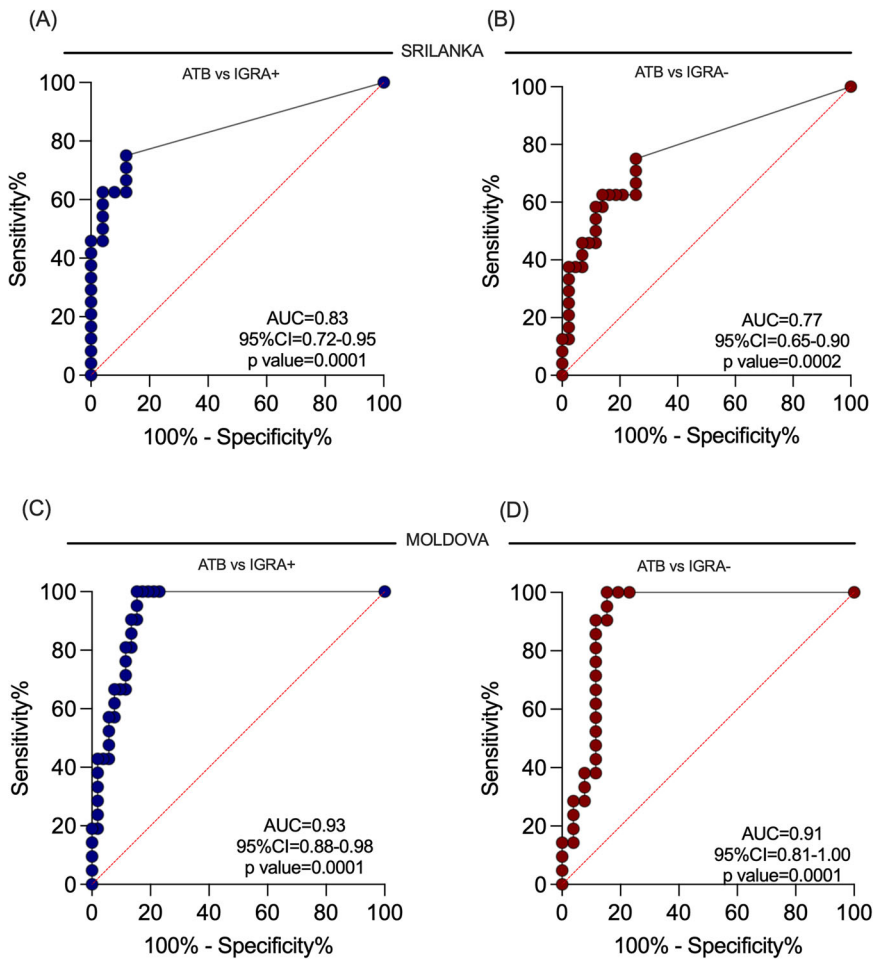


Fig. 6 | The diagnostic potential of ATB116. Receiver Operating Characteristic (ROC) curves for Sri Lanka (A, B) and Moldova (C, D). ATB vs. IGRA+ (A, C) and ATB vs. IGRA- (B, D). The x-axis shows the false positive rate (100%-specificity%), and the y-axis shows the true positive rate (sensitivity %). Each point on the curve

represents a different threshold value used to calculate the true and false positive rates. The area under the curve (AUC) is shown for predictive performance, including the confidence interval (CI) and associated two-sided p-value. Source data are provided as a Source data file.

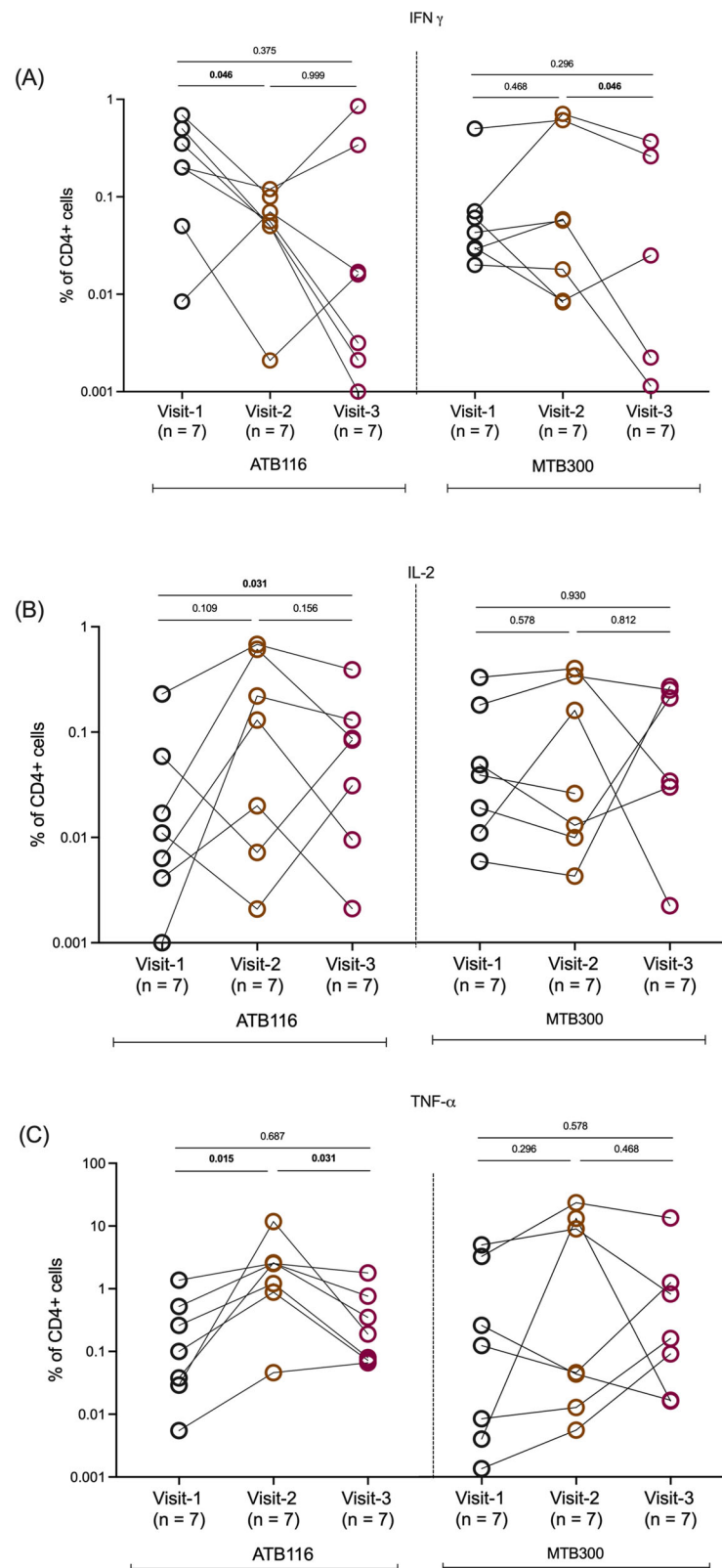


Fig. 7 | Characterization of ATB116- and MTB300-specific CD4 T cell responses. **A–C** Frequency of cytokine-producing, IFN γ (**A**), IL-2 (**B**), and TNF α (**C**), CD4 T cells in response to ATB116 and MTB300 in longitudinal samples from patients with ATB (visit 1; at diagnosis, visit 2; mid-treatment, visit 3; end of treatment, $n = 7$). Each

point represents one participant, median $\pm$ interquartile range is shown. Wilcoxon signed rank test (two-sided) was used. Source data are provided as a Source data file.

The previous work identifying epitopes from IGRA+ participants led to the development of a peptide “megapool”, MTB300, which allows for capturing diverse *Mtb*-specific responses irrespective of the HLA alleles expressed in the population⁴². Using the same multi-epitope approach here, we defined a new megapool, ATB116, which contains epitopes that are not included in MTB300 and have only been found to be reactive in participants with ATB. Specifically, ATB116 could discriminate ATB at diagnosis and mid-treatment from control participants who were IGRA+ and IGRA-, in geographically diverse cohorts from Sri Lanka and Moldova. There was a higher frequency of reactivity against ATB116 in IGRA+ and IGRA- controls from Moldova compared to Sri Lanka. This could be because the Moldovan cohort of IGRA +/- are household contacts within 6 months of a primary ATB index case and have thus been recently exposed to *Mtb*. Further studies will determine whether this reactivity is simply a measure of recent exposure or whether these individuals will develop active TB in the future. In addition, future studies will determine whether responses against the ATB116 epitopes are protective or pathological. We observed a decrease in IFN γ response upon treatment, suggesting that antigen expression levels change during treatment as it takes effect. This and the ATB-specific recognition may indicate that these peptides are not protective. However, no firm conclusions can be drawn from the results presented here. No IL-17 was detected in the individuals with ATB mid-treatment, but this does not rule out differences between IFN γ and IL-17 at diagnosis of ATB, where we have not measured IL-17. Contradictory findings have been reported for the role of polyfunctional CD4 + T cells in relation to host containment of *Mtb* infection. On one hand, stronger polyfunctional *Mtb*-specific CD4 + T cell responses have been observed in adults with acid-fast (AFB) smear negative sputum samples and latent TB infection (LTBI) compared to those with AFB smear positive tuberculosis (TB)^{57–59}. Consistent with our observations, these studies have also reported a greater proportion of T cells producing TNF α , IFN γ , IL-2, and TNF α responses were similar when cells were stimulated with the peptide pools corresponding to different protein categories. In the longitudinal samples, IFN γ decreased from diagnosis to the second time point 2 m post diagnosis, TNF α increased, and IL-2 showed a trend for an increase in response to ATB116. Successful TB treatment, which reduces the bacterial load, is also associated with significant increases in the proportion of polyfunctional CD4 + T cells. On the other hand, there are studies demonstrating a positive correlation between polyfunctional CD4 + T cell responses and increased bacillary load, showing stronger responses in adults with TB compared to those with LTBI and healthy household contacts^{60,61}. These contradictory results highlight the limitations of correlative studies in determining whether the polyfunctionality of CD4 + T cells plays a causal role in the immune control of the pathogen or reflects the underlying bacterial burden. We have previously described a gene signature in individuals with latent TB at risk of developing active TB, which overlaps with a signature from ATB⁶². This gene signature approach, together with ATB116-specific reactivity, may be useful for identifying individuals at risk of developing active TB. The differences in sensitivity observed between the Moldovan and Sri Lankan cohorts may reflect differences in the infecting *Mtb* strains, differential exposure to NTM and other environments or infections, and potential host factors. Taken together, further exploration of the ATB116 pool for its diagnostic potential across the spectrum of *Mtb* infection is warranted. Ideally, prospective studies to investigate whether reactivity to ATB116 can predict progression to ATB. In addition, ATB116, or versions thereof, may be useful as a whole blood assay in areas with high levels of IGRA positivity where there is a need to selectively identify ATB.

We could not detect a functionally distinct immune response in terms of multifunctionality. The majority of the responding T cells, irrespective of the functional categories, produced a single cytokine, with the majority producing TNF α . Some differences were observed

regarding memory phenotype, where the cell wall and cell processes category had a higher frequency of effector memory T cells than the other categories. This suggests that T cell responses against these epitopes result in increased differentiation of the responding memory subset⁶³. Overall, we observed that epitope-specific T cells were predominantly CD45RA-CCR7- effector memory cells, followed by CD45RA-CCR7+, which agrees with previous studies in patients with ATB^{64–67}. Taken together, it has previously been shown that differentiation of T cells towards later-stage effector memory during ongoing antigen expression primarily favors the expression of IFN γ and/or TNF α ^{63,68}, as observed here. Ongoing studies are investigating the ATB116 and other epitope-specific T cell phenotypes and subsets in more detail, including the involvement of Th1^{22,62,69,70}.

In conclusion, this study addressed a critical knowledge gap in understanding the diversity of *Mtb* antigens capable of eliciting differential T cell responses during various stages of infection by identifying *Mtb*-derived T cell epitopes in patients with active tuberculosis (ATB) using a proteome-wide library with over 21,000 peptides, including TB vaccine candidate antigens. As a result, a novel multi-epitope “ATB116” pool was created, capable of discriminating ATB from IGRA+ and IGRA- individuals, offering the potential use in diagnostic tests and measuring *Mtb*-specific immune responses.

Methods

Study approval

All participants provided written informed consent for participation in the study. Ethical approval was obtained from the institutional review boards at La Jolla Institute for Immunology (LJI; Protocol Numbers: VD-090, VD-143, VD-175), Universidad Peruana Cayetano Heredia (66754), Phthiopneumology Institute (CE-3/2018), University of California San Diego (180068), and University of Colombo for General Sir John Kotelawala Defense University, Sri Lanka (EC18-122, EC15-094). Results for IGRA+ individuals from San Diego, USA (Fig. 2D) and the Western Cape region, South Africa (Fig. 3B) are included here for comparison purposes. They have been reported previously; San Diego cohort²², and South African cohort⁴².

Study participants

We recruited a total of 184 participants over 18 years of age for this study from three different cohorts, UPCH in Peru, the General Sir John Kotelawala Defense University in Sri Lanka, and the Phthiopneumology Institute in the Republic of Moldova. From Peru, 21 participants with ATB who were mid-treatment (3–4 months post-diagnosis) were recruited from 2012 to 2013. The cohort consisted of 62% males and 38% females. From Sri Lanka, a cohort of patients with ATB (at diagnosis, $n = 24$; 96% males and 4% females), IGRA+ individuals ($n = 25$; 48% males and 52% females), and IGRA- individuals ($n = 43$; 39% males and 60.5% females) were recruited from 2019 to 2022. A subset of the patients with ATB ($n = 7$) was followed longitudinally from the time of diagnosis until the end of treatment. They provided blood samples at diagnosis, 2 months post-diagnosis, and 6 months post-diagnosis. From Moldova, a cohort of patients with ATB (at diagnosis, $n = 9$), ATB (mid-treatment, $n = 12$), IGRA+ individuals ($n = 52$), and IGRA- individuals ($n = 26$) were recruited from 2018–2022 (no biological sex information available). The IGRA+ and IGRA- individuals were household contacts of a patient with active TB (e.g., an “index case”). They provided blood samples up to 6 months after the index case received their ATB diagnosis.

Healthy participants were classified into IGRA+ (i.e., Latent TB infection) and IGRA- groups based on IGRA tests (QuantiFERON-TB Gold Plus, Cellestis and/or T-spot.TB, Oxford Immunotec). Individuals with ATB had symptomatic pulmonary TB, diagnosed by a positive GenXpert (Cepheid, Inc.), positive sputum smear, and/or a positive culture.

Participants with pulmonary TB recruited in Peru were aged 18–50, had documented culture confirmed TB from sputum, and were currently 3–4 months post-diagnosis of ATB. They responded well to treatment and had regained any weight lost due to the infection. Individuals with MDR or XDR-TB, and those diagnosed HIV, HBV or HCV infection were excluded, as well as patients with significant systemic diseases, including, for example, diabetes, renal disease, liver disease, uncontrolled hypertension, and malignancy. They provided 100 ml leukapheresis samples.

PBMC isolation and thawing

PBMCs were obtained by density gradient centrifugation (Ficoll-Hypaque, Amersham Biosciences) from leukapheresis or whole blood samples, according to the manufacturer's instructions. The PBMC processing at the site in the Republic of Moldova used SepMate tubes (StemCell). Cells were resuspended in FBS (Gemini Bio-Products) containing 10% DMSO (v/v, Sigma) and cryopreserved in liquid nitrogen.

Cryopreserved PBMC were quickly thawed by incubating each cryovial at 37 °C for 2 min, and cells transferred to cold medium (RPMI 1640 with L-glutamin and 25 mM HEPES; Omega Scientific), supplemented with 5% human AB serum (GemCell), 1% penicillin streptomycin (Life Technologies), 1% glutamax (Life Technologies) and 20 U/ml benzonase nuclease (MilliporeSigma). Cells were centrifuged and resuspended in complete RPMI medium to determine cell concentration and viability using trypan blue. All samples had a > 60% viability.

Peptide screening and peptide pool preparation

The present study screened a total of 21,220 peptides. These peptides include the same library that was screened in IGRA+ participants of 20,610 *Mtb* peptides (2 to 10 per ORF, average 5), including 1660 variants not totally conserved amongst the selected *Mtb* genomes: five complete *Mtb* genomes (CDC1551, F11, H37Ra, H37Rv and KZN 1435) and sixteen draft assemblies²². Along with these peptides, 610 peptides were also selected, which include 93 peptides that are not found in *Mtb* but present in the *Mycobacterium bovis* BCG strains Mexico, Tokyo 172, and Pasteur 1173P2, and 517 peptides that are 15-mers overlapping by ten amino acids spanning the entire sequence of 12 TB vaccine candidate and IGRA antigens. The vaccine candidates included ID93: GLA-SE (Rv3619c, Rv3620c, Rv1813, and Rv2608), H1:IC31 (ESAT-6 and Ag85B), H4:IC31 (Ag85B, TB10.4), H56:IC31 (Ag85B, ESAT-6, and Rv2660c), M72/AS01E (Mtb32A, PPE18), and three candidates with Ag85A alone (Ad5 Ag85A, ChAdOx1-85A/MVA85A, and MVA85A). The IGRA antigens include ESAT-6, which is also a vaccine candidate antigen, and CFP10.

The peptides were synthesized as crude material on a small (1 mg) scale by Mimotopes (Australia). The peptides were solubilized using DMSO at a concentration of 20 mg/ml. The peptides were pooled into peptide pools. The previous peptide library was pooled into 1036 peptide pools of about 20 peptides each (average 19.9 ± 0.5). The 93 non-*Mtb* peptides were pooled into 5 peptide pools. The 15-mer peptides overlapping by 10 amino acids spanning the TB vaccine candidate and IGRA antigens were pooled into 26 peptide pools. Thus, from the 21,220 total peptides, a total of 1067 peptide pools were made.

Individual peptides were mixed in equal amounts after being dissolved in DMSO for the megapools (PC85, PC71, ATB116, and MTB300), as described previously⁴². Each peptide pool was then placed in a lyophilizing flask and subjected to lyophilization for 24 h. The resulting semi-solid product was dissolved in water, frozen, and lyophilized again until only solid product remained. This process was repeated several times until only solid product remained after lyophilization. Finally, the peptide pool was re-suspended in DMSO at a higher concentration per peptide (0.7 mg/ml per peptide) than before lyophilization, to reduce the concentration of DMSO in the assays.

Ex vivo IFN γ and IL-17 fluorospot assay

IFN γ and IL-17 production was measured by a Fluorospot assay with all antibodies and reagents from Mabtech (Nacka Strand, Sweden). Plates were coated overnight at 4 °C with an antibody mixture containing mouse anti-human IFN γ (clone 1-D1K) and mouse anti-human IL-17 (clone MT44.6). Briefly, 2×10^5 PBMCs were added to each well of pre-coated Immobilon-FL PVDF 96-well plate in the presence of peptide pools at a concentration of 2 μ g/ml, individual peptides at 5 μ g/ml, PHA at 10 μ g/ml (positive control) and media containing DMSO (amount corresponding to percent DMSO in the pools/peptides, as a negative control). Plates were incubated at 37 °C in a humidified CO₂ incubator for 20–24 h. All conditions were tested in triplicate, except the negative control, which was tested in six individual wells. After incubation, plates were developed according to the manufacturer's instructions. Briefly, cells were removed and wells were washed with PBS/0.05% Tween 20 using an automated plate washer. After washing, an antibody mixture containing anti-IFN γ (7-B6-1-FS-BAM) and anti-IL-17 (MT504-WASP) prepared in PBS with 0.1% BSA was added to each well and plates were incubated for 2 h at room temperature. The plates were again washed and incubated with diluted fluorophore-conjugated anti-BAM-490 and anti-WASP-640 antibody for 1 h at room temperature. Finally, the plates were washed and incubated with a fluorescence enhancer for 15 min, blotted dry and fluorescent spots were counted by computer-assisted image analysis (IRIS Fluorospot reader, Mabtech, Apex version 1.1.59.128, Sweden).

Each pool or peptide was considered positive compared to the background that had an equivalent amount of DMSO based on the following criteria: (i) 20 or more spot-forming cells (SFC) per 10^6 PBMC after background subtraction, (ii) a greater than 2-fold increase compared to the background, and (iii) $p < 0.05$ by student's t-test or Poisson distribution test when comparing the peptide or pool triplicates with the negative control. The response frequency was calculated by dividing the number of participants responding by the no. of participants tested. The magnitude of response (total SFC) was calculated by summation of SFC from responding participants.

Intracellular cytokine staining assay

PBMC at 1×10^6 per condition were stimulated with peptide pools (2 μ g/ml) for 18–20 h in complete RPMI medium at 37 °C with 5% CO₂. PBMCs incubated with DMSO at the percentage corresponding to the amount in the peptide pools were used as a negative control to assess nonspecific or background cytokine production, and anti-CD3/CD28 (1 μ g/ml; OKT3 and CD28.2) stimulation was used as a positive control. For chemokine receptor staining, antibodies were added during the stimulation. After 18 h, 2.5 μ g/ml each of BFA and monensin was added for an additional 5 h at 37 °C. Cells were then harvested and incubated in a blocking buffer containing 10%FBS and 1 μ g/mL Human Fc block (BD Biosciences, USA) for 20 min at 4 °C. Next, cells were stained using fixable live/dead stain for 20 min at room temperature and then stained with surface-expressed antibodies; (Supplementary Table 1) diluted in FACS buffer and IX Brilliant Stain Buffer (BD Biosciences, USA) for 20 min at room temperature. Cells were permeabilized and fixed for intracellular cytokine staining using cytoperm fixation buffer (Biolegend) for 20 min at room temperature. After incubation, cells were stained for cytokines (IFN γ , IL-2 and TNF α) for 20 min at room temperature. Samples were acquired on a ZE5 cell analyzer (BioRad). Frequencies of CD4 or CD8 T cells responding to each peptide pool were quantified by determining the total number of gated CD4 or CD8 and cytokine-producing cells and background values subtracted (as determined from the negative control) using FlowJo X Software. Combinations of cytokine-producing cells were determined using Boolean gating. The lower limit of detection for the frequency of cytokine-producing CD4 T cells after background subtraction was set to 0.001%.

Statistical analysis

Statistical analyses were performed using GraphPad Prism software (GraphPad Software, Inc., San Diego, CA, USA, version 9.2). Data is shown as median with interquartile range. Non-parametric test was applied after checking for normality. A two-tailed Mann-Whitney U test was used for comparison between two groups. Wilcoxon signed-rank tests were used for the comparison of cytokine-producing cells in longitudinal samples from patients with ATB. p -value ≤ 0.05 was considered significant. No statistical method was used to predetermine the sample size. No data were excluded from the analysis. The experiments were randomized. The investigators were blinded to cohort allocation during the experiment setup and unblinded during analysis.

Reporting summary

Further information on research design is available in the Nature Portfolio Reporting Summary linked to this article.

Data availability

The identified epitopes from the proteome-wide screen in participants with ATB have been submitted to IEDB (Immune Epitope Database; www.iedb.org) and can be found under the submission ID: 1000914. H37Rv genome was used as reference genome (GenBank accession number NC_000962) for comparing protein categories between identified antigens and overall in the *Mtb* genome. The data generated in this study are provided in the Supplementary information and Source Data file. Source data are provided with this paper.

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Author contributions

C.S.L.A., A.S., and B.P. participated in the design and direction of the study. S.P., J.M., C.C., and C.S.L.A. performed and analyzed the experiments. M.S., R.H.G., N.C., V.C., D.G.C., A.C., T.R., J.S.B.P., T.C., B.G., and A.D. DS, recruited participants, performed clinical evaluations, and isolated PBMCs. C.S.L.A. and S.P. wrote the manuscript. All authors read, edited, and approved the manuscript before submission.

Funding

This work was supported by the National Institutes of Health contract HHSN272200900044C (to A.S.), 75N93019C00067 (to B.P. and C.S.L.A.), and R01 AI137681 (to A.C.). The funders had no role in study design, data collection, analysis, decision to publish, or manuscript preparation.

Competing interests

The authors declare no competing interests.

Additional information

Supplementary information The online version contains supplementary material available at <https://doi.org/10.1038/s41467-024-45058-9>.

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Peer review information *Nature Communications* thanks Susanna Brighenti, Markus Maeurer and the other anonymous reviewer(s) for their contribution to the peer review of this work. A peer review file is available.

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A Nine-Gene Blood-Based Signature Meets the World Health Organization Target Product Profiles for Diagnosis of Active Tuberculosis and Predicting Progression from Latent to Active Disease

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Rationale: The World Health Organization (WHO) reported that approximately 10 million people developed active tuberculosis (ATB) in 2020, of which only 5.8 million were diagnosed and 1.5 million patients died. The current reference-standards for diagnosis of ATB: sputum culture and smear microscopy, have several limitations, including low sensitivity, difficulty in obtaining sputum samples, and inability to predict progression from latent TB (LTB) to ATB, highlighting a need for non-sputum-based diagnostics. As part of its End TB strategy, the WHO has identified the need for non-sputum-based tests that meet target product profiles (TPP) of 90% sensitivity and 70% specificity for diagnosis of ATB and 75% sensitivity and specificity for predicting progression from LTB to ATB. Although several single-cohort studies have demonstrated promising blood-based gene expression signatures for diagnosis of ATB, they often fail to generalize to larger patient populations with more biological and clinical heterogeneity. However, a 3-gene blood-based signature, identified using diverse datasets, has been successfully translated into a prototype point-of-care test that meets the WHO TPPs. The successful translation of the 3-gene signature demonstrated the power of integrating large amounts of heterogeneous data to identify robust, generalizable disease signatures. We hypothesized that including more diverse datasets comprising patients with ATB or other lung and inflammatory diseases, would identify novel, highly robust host response-based signatures for diagnosis of ATB and predicting progression from LTB to ATB that meet performance expectations of the WHO TPP. **Methods:** We used a multi-cohort analysis framework to integrate and analyze data from 3,615 peripheral blood samples in 49 publicly available transcriptomic datasets (discovery cohorts), from healthy controls and patients with LTB, ATB, and other diseases (COPD, viral infections, sarcoidosis, lung cancer, etc.). We then used (1) 3,836 blood samples from 28 retrospective datasets and (2) 334 prospectively collected blood samples from Moldova as validation cohorts. **Results:** Using the discovery cohorts we identified a 9-gene signature, which classified patients with ATB with 90.1% sensitivity and 81.7% specificity in retrospective validation cohorts and with 90.2% sensitivity and 68.9% specificity in the prospective Moldova cohort (Figure 1A-B). In a longitudinal cohort of adolescents, the 9-gene signature predicted progression from LTB to ATB up to 1 year prior to sputum conversion with 76% sensitivity and 83.3% specificity (Figure 1C). Finally, it also correlated with lung inflammation in the Catalysis Treatment Response Cohort (Figure 1D). **Conclusion:** Overall, the 9-gene signature meets the WHO TPPs required for the End TB strategy.

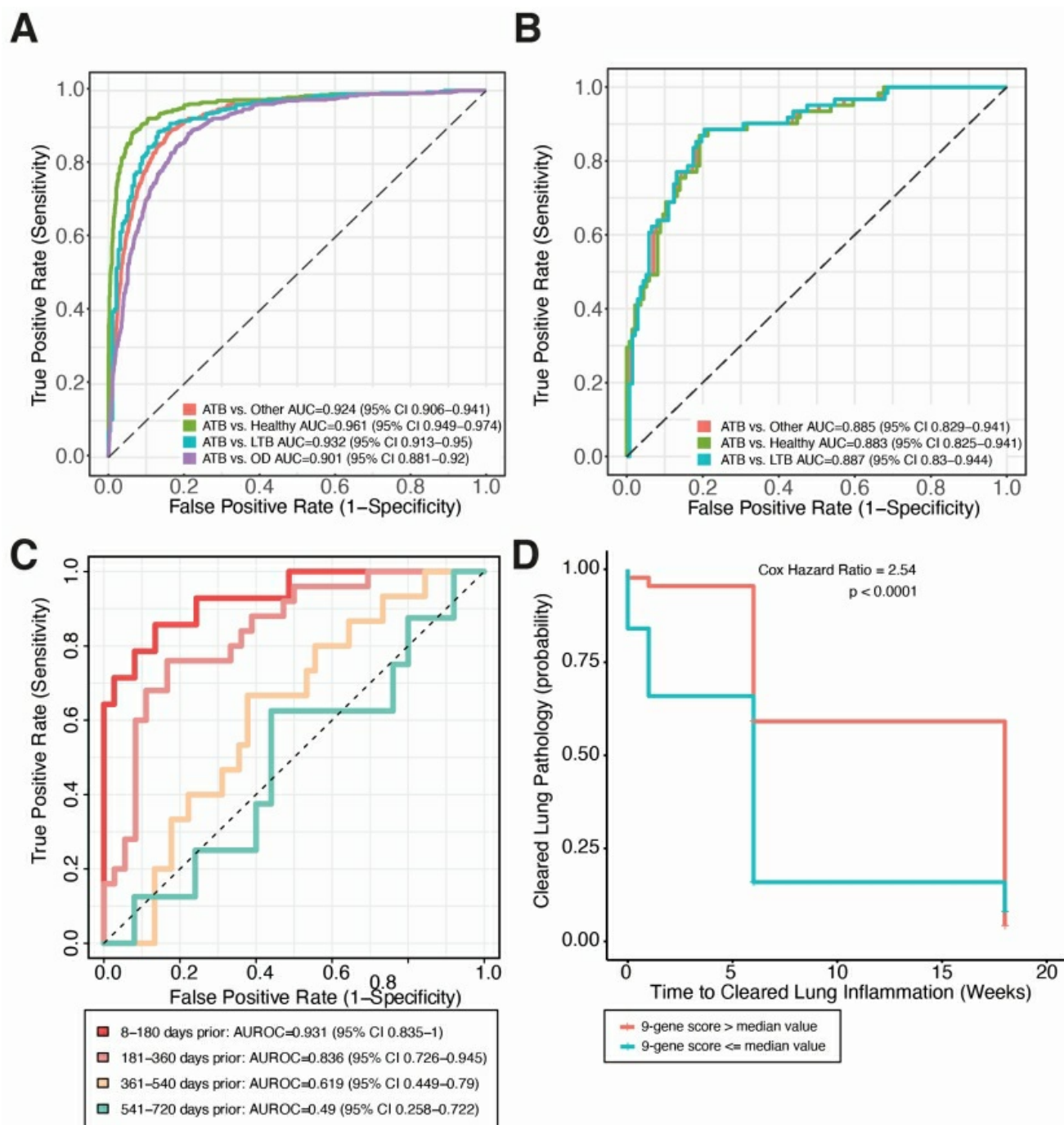


Figure 1. 9-gene signature validates in independent retrospective and prospective cohorts.

(A) ROC curves for comparing ATB vs. all other samples (Other), or individually comparing ATB vs. Healthy, LTB or Other Disease (OD) samples, if present, in independent validation and (B) a prospective Moldova cohort. (C) ROC curves comparing progressor and non-progressor samples collected at different time points in the ACS cohort. (D) A Cox regression analysis shows that patients with a 9-gene score higher than the median value have a significantly higher probability of cleared lung pathology across all time points.

All ROC curves are obtained with The 9 Gene score, which is computed as the difference of geometric means of the upregulated and downregulated genes.

TB, tuberculosis; ATB, active tuberculosis; LTB, latent tuberculosis; ROC, receiver operating curve; AUROC, area under the receiver operating curve; ACS, Adolescent cohort study.

This abstract is funded by: Bill & Melinda Gates Foundation OPP1113682; DoD USAMRAA Award W81XWH-18-1-0253