Are mRNA based transcriptomic signatures ready for diagnosing tuberculosis in the clinic? - A review of evidence and the technological landscape

Yohhei Hamada,[a,1] Adam Penn-Nicholson,[b,1] Sriram Krishnan,[b] Daniela Maria Cirillo,[c] Alberto Matteelli,[d] Romain Wyss,[b] Claudia M. Denkinger,[b,e,f] Molebogeng X. Rangaka,[a,g] Morten Ruhwald,[b] and Samuel G. Schumacher[b]*

[a]Insitute for Global Health, University College London, London, United Kingdom
[b]FIND, Geneva Switzerland
[c]IRCCS San Raffaele Scientific Institute, Milan, Italy
[d]Collaborating Centre for TB/HIV Co-infection and TB Elimination, Dept of Infectious and Tropical Diseases, University of Brescia, Brescia, Italy
[e]Division of Tropical Medicine, Centre of Infectious Disease, Heidelberg University Hospital, Heidelberg, Germany
[f]German Center of Infection Research, Partner Site Heidelberg University Hospital, Heidelberg, Germany
[g]School of Public Health, and Clinical Infectious Disease Research Institute-AFRICA, University of Cape Town, South Africa

Summary

Advances in discovery and validation of diagnostic, prognostic and treatment-monitoring transcriptomic signatures of tuberculosis (TB) disease could accelerate the goal to end TB. We conducted a review to evaluate whether mRNA transcriptomics technologies are sufficiently mature to develop accurate next-generation TB diagnostic tests. Early studies tended to be limited in sample size, diversity of population groups, sample collection and processing methods, while recent prospective studies have addressed these limitations. Some of the existing signatures could be used for triage; however, high cost and complexity could limit their use. For a confirmatory test, setting an optimal cut-off to maintain specificity across populations and settings is a challenge. mRNA signatures have shown the potential to quantitatively monitor response to treatment. No prognostic signatures can accurately predict progression to active TB over 2 years while short term prediction is possible. The management strategy should be defined for individuals with positive prognostic tests.

Introduction

Despite advances in discovery and validation of diagnostic, prognostic and treatment-monitoring signatures of tuberculosis (TB) disease, coupled with rapid advances in assay development and deployment capabilities, progress in reducing morbidity and mortality from TB has been slow. In 2020, there were an estimated 9·9 million cases of TB resulting in 1·3 million deaths, with disruptions caused by COVID-19 leading to a huge drop in notifications from 7·1 million in 2019 to 5·8 million.[1]

Currently, the diagnosis of active TB mainly relies on the detection of the *Mycobacterium tuberculosis (M.tb)* bacilli itself in sputum, a relatively inaccessible specimen associated with advanced pulmonary TB disease. In fact, up to half of all TB cases do not report symptoms (i.e. subclinical TB disease) and it is increasingly appreciated that undetected subclinical TB disease is responsible for a large part of TB transmission.[2,3] Furthermore, a large burden of TB related deaths is accounted for by infants, children and immune-compromised adults, who may not have a productive cough and lack cavitation, making the diagnosis of paucibacillary or extrapulmonary TB extremely difficult. Currently available tests that detect the presence of bacterial antigens such as lateral flow urine lipoarabinomannan

*Corresponding author at: FIND, Campus Biotech Building B2, Level 0, 9 Chemin des Mines, Geneva, Switzerland.
E-mail addresses: samuel.schumacher@finddx.org, schumacher.samuel@gmail.com (S.G. Schumacher).
[1] First two authors contributed equally.

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assay (LF-LAM) are useful only in detecting TB in severely immunocompromised patients, particularly those with advanced HIV disease.[4] There is no World Health Organization (WHO) endorsed blood-based tests to diagnose active TB. Thus, there is an urgent need to develop a new class of TB diagnostic tests that can overcome the limitations of existing tests.

TB infection is currently diagnosed by immune sensitisation tests such as the tuberculin skin test (TST), skin tests specific to TB (e.g. Diaskintest [Generium, Russia],[5] C-Tb [Serum Institute of India, India][6] and EC skin test [Anhui Zhifei Longcom, China])[7,8] and interferon-gamma release assays (IGRA).[8] These assays fail to differentiate active TB from infection and do not allow reliable prediction of future development of active TB.[9] The WHO End TB strategy outlined the need for such a test that can detect the presence of incipient TB, a state with viable TB bacilli prior to manifestation of disease.[10]

For treatment monitoring, the currently used tests are insufficient due to the low sensitivity of smear microscopy, the detection of killed/dead bacilli by molecular tests, and the delayed time to positivity for culture.[11] The novel drug-resistant TB treatment guidelines put several drugs into the first-line drugs of choice for which currently no commercial drug-sensitivity testing is available;[12] hence, improvements in treatment monitoring have become ever more important. Further, many patients could probably be cured with less than six months of treatment but in the absence of an accurate treatment monitoring marker, we cannot differentiate them from those who require a full course of treatment.[13]

A new class of diagnostic technologies exists. These use parts of the host transcriptome to detect disease states or to even predict potentially life-threatening conditions. Typically these technologies measure the expression levels of mRNAs, derived from blood samples, reflecting the genes that are actively being expressed at a given time. Analysis of the host transcriptome can reveal important clues about gene regulation in response to biological conditions in the organism. Some of these techniques have also been commercialised and are being used in cancer diagnostics (e.g. OncotypeDX [Exact Sciences, USA], MammaPrint [Agendia, USA]).[14] These transcriptomic signatures were validated through clinical trials for predicting patients who benefit from chemotherapy and have been endorsed in clinical guidelines. Studies suggest that gene expression signatures can be used in TB to detect active TB, distinguish active TB from other infectious diseases or other disease states and herewith predict TB disease risk and monitor treatment response.[15-20]

We carried out a narrative review to evaluate whether transcriptomic technologies, specifically detection of mRNA transcripts, were sufficiently mature to develop accurate next-generation TB diagnostic tests by benchmarking against WHO defined performance targets. Additionally, we surveyed the current and emerging technology landscape that would facilitate the development of transcriptomics-based point-of-care (PoC) test diagnostics devices, including technologies for sample collection, processing of biosamples and quantification of mRNA expressions. Our review focused on using blood as the diagnostic sample to extract mRNA based on the ease of collection for all patient cohorts.

Transcriptomics

Transcriptomic profiling is a powerful tool for unbiased discovery of new TB diagnostic, prognostic and treatment response signatures. Discovery platforms commonly in use are microarrays, quantitative reverse transcriptase PCR (qRT-PCR), nano-string and RNA-Seq. Once a subset of genes that could classify disease states and pathogens are identified, its performance has to be validated on independent cohorts. Finally, for clinical use, these gene sets will have to be tested on a broader and representative population and evaluated for diagnostic accuracy through clinical trials.

Various types of clinical samples have been used for transcriptomic analysis. However, at present, mRNA derived from blood appears to be the most promising format for transcripts based TB diagnostics. While peripheral blood mononuclear cells (PBMCs) yield high-quality RNA, isolating PBMC from whole blood involves multiple steps and requires trained personnel and equipment. Hence, whole blood samples are preferable for PoC TB diagnostics.

Blood is typically collected from a venous puncture or a finger prick. Venous blood provides greater volume, and thus greater yield of RNA, but is more invasive and requires trained professionals, often lacking in low resource settings compared to the fingerprick process. These advantages make blood from finger prick the desirable format for any PoC diagnostics to be used in under-resourced settings.

The heterogeneity of *M.tb* infection and disease within individuals and across populations

TB is a spectrum ranging from quiescent controlled infection to active local or disseminated disease. Even in individuals with microbiologically-confirmed cure, hotspots representing *M.tb* growth in the lungs are still evident.[21] Further, otherwise healthy individuals without symptoms can have subclinical TB.[21-23] There is a clear need for differentiating between different states allowing earlier diagnosis of those with active disease for effective treatment and targeted investigation of populations at risk for progression to active TB for TB preventive treatment (TPT).

But this spectrum in disease state places clear limitations in study design using the appropriate characterisation of TB disease vs. infected controls who are healthy or have other diseases.[24] TB-infected individuals living in a highly endemic TB region such as South Africa[15] are likely inherently different to those living in the UK[25,26] or the US[27] or even other endemic settings such as the Gambia[28] where different *M.tb* and non-tuberculous mycobacterial strains are in circulation and the frequency of TB exposure is different. Yet, any effective signature to be adopted for product development should ideally have cross-demographic application — otherwise, low production costs are unlikely to be achieved.

Additionally, any signature intended to measure treatment response should ideally be evaluated on independent validation cohorts with similar characteristics for duration of treatment and method of *M.tb* detection.

Potential applications of of mRNA signatures and their target product profiles

There are four potential applications of mRNA signature-based diagnostic tests: triage, diagnosis, prediction and treatment monitoring.

The WHO defined Target Product Profiles, through expert consultations, outlining recommended minimum requirements for any new high priority diagnostics for TB (Table 1).[29] The report called for a non-sputum based diagnostic test for all TB to initiate TB treatment in levels below the microscopy centre. A low-cost triage test was also considered important, which can be used at the first encounter to distinguish between individuals who need confirmatory testing from those in whom TB has been ruled out. Such tests can be used for example in systematic TB screening in community settings. The minimal requirements for a non-sputum based triage test were defined as 90% sensitivity and 70% specificity to act as a rule-out test. On the other hand, diagnostic or confirmatory tests need higher specificity (> 98%).

Tests for prediction are intended to identify individuals who will develop active TB and thus need TPT. The minimal targets for sensitivity and specificity are both 75% for progression to active TB in the next 2 years.[10] A period of 2 years was chosen because about half of incident TB after infection occurs within 2 years and an early study on an mRNA signature showed a substantial decline in its predictive performance after 2 years.[15,30] The evaluation of predictive performance needs longitudinal studies.[30] Additionally, trials testing a treatment strategy based on test results are essential to demonstrate the impact of such tests on clinical outcomes.

Tests for treatment monitoring can be used during treatment to identify those who are not cured and hence at risk for relapse. The results are expected to be correlated with standard bacteriological measures of treatment success (e.g. culture results). The use of such tests can help individualise the treatment duration; treatment can be shortened or extended guided by the tests. Further, the use of the tests as surrogate endpoints would facilitate the clinical trials of new regimens. Tests for treatment monitoring need to be evaluated in longitudinal studies to demonstrate their performance to predict relapse-free cures.

Triage and diagnosis of active TB

One of the earliest blood-based gene expression signatures capable of differentiating active TB from TB infection or recently cured TB was published by Mistry et al.[31] The authors identified nine genes that could discriminate between people with active, latent, recurrent, and cured TB. A seminal study identified a 393 gene signature,[25] dominated by a neutrophil-derived type 1 IFN response, that differentiated active TB from TB infection and other diseases, and was reduced after initiation of anti-TB treatment.[26] Since then multiple studies have evaluated TB diagnostics based on the expression of IFN-inducible genes, and the number of genes have gradually reduced to as few as 1 gene.[32−35] Nonhuman primate studies have also identified up-regulation of IFN inducible genes after early infection events, with a correlation in disease pathology and lung inflammation as measured by PET-CT.[36,37] In people living with HIV with TB, Esmail et al showed the enrichment of transcripts related to the classical complement pathway and Fcγ receptors.[38] On the other hand, the background level of transcripts related to IFN signaling was high in people living with HIV who were not on antiretroviral therapy, resulting in less discriminatory power to identify sub-clinical TB.

Across all such studies, the variability in study design and TB case definitions, along with sampling methodology, duration of TB disease and/or treatment, demographic, geographic and strain diversity and differences in technical tools across and within different methodologies (even differences in lot-to-lot variation of microarrays) has contributed to a multitude of signatures with limited overlap of identified genes. To this end, Sweeney et colleagues conducted a multi-cohort analysis using 14 publicly available RNA-Seq and microarray datasets containing over 2500 samples, including adult and pediatric samples, from 10 different countries, inclusive of those beyond Africa. They used three data sets containing 1023 samples, to derive a "TB Score" (as in Anderson et al, 2014[33]) using a set of 3-genes (GBP5, DUSP3, KLF2) that could discriminate active TB from healthy controls, TB infection and other diseases. Further, this 3-gene set was not confounded by HIV status, drug resistance or bacille Calmette-Guérin (BCG) vaccination. One limitation of this study is the inherent variability between different batches of

Use case	Goal of the test	Potential applications	Characteristics	Optimum	Minimum
Triage	To identify individuals who need a confirmatory test or those who are unlikely to have TB	• Systematic TB screening in community settings. • Ruling out active TB before TB preventive treatment	Sensitivity Specificity Cost	> 95% > 80% < US$ 1.00/ test	> 90% > 70% < US$ 2.00/ test
Diagnosis	To diagnose both pulmonary and extrapulmonary TB using non-sputum samples for the purpose of initiating treatment	• Diagnosis of TB and treatment initiation on the same day by health workers with limited training in peripheral facilities	Sensitivity for pulmonary TB in adults	≥ 98% for smear-positive culture-positive pulmonary TB ≥ 68% for smear-negative culture-positive pulmonary TB	Overall sensitivity should be ≥ 65% but should be > 98% among patients with smear-positive culture-positive pulmonary
			Sensitivity for extrapulmonary TB in adults	>=80%	No lower range of sensitivity defined
			Sensitivity in children	>= 66%	No lower range of sensitivity defined
			Specificity	>= 98%	
			Cost	< US$ 4.00/ test	< US$ 6.00/ test
Prediction for progression to active TB	To identify people who develop active TB and thus benefit from TB preventive treatment	• To identify individuals with or without risk factors who are at risk for TB development and should be given TB preventive treatment	Sensitivity for progression to active TB in 2 years	>=90%	>=75%
			Specificity for progression to active TB in 2 years	>=90%	>=75%
			Cost	< US$ 5.00/ test	< US$ 10.0-100/ test
Monitoring treatment response	To identify people who are not cured and at risk for relapse.	• Individualise treatment duration • Evaluate the efficacy of new drugs quickly.	No target product profile is available		

***Table 1*: Test characteristics for new TB diagnostics set by the world health organization.**
Note: We focused on the performance of the tests and costs, which are considered critical attributes of the tests. See full target product profiles and explanations in WHO reports.[10,29]

microarray runs, and certainly across different studies performed in different institutes with different kits and equipment (e.g. Illumina and Affymetrix). Certainly, appropriate transformation and normalisation techniques (e.g. quantile normalisation, distance weighted discrimination) need to be applied to limit noise and allow cross-platform integration.

Warsinske et al evaluated 16 gene signatures identified through a systematic review including Sweeney3 using 24 data sets (n=3083).[20] The study found that the Sweeney3 and Sambarey10 signatures had the highest accuracy, with a specificity of 74% at 90% sensitivity in datasets of culture-confirmed TB, meeting the WHO target for a triage test. However, when evaluating them in all data sets including all TB regardless of the method of diagnosis, the specificity of Sambarey10 declined to 59% while that of Sweeney declined less (to 66%). The authors pointed out that the observed lower specificity may be underestimated because of the limited diagnostic performance of reference TB tests. The review excluded the 16-gene signature discovered by Zak et al and the 4-gene signature by Suliman et al that were originally designed for predicting the development of active TB, both of which are found to have excellent performance as a triage test as described below.[15,28,39]

Another systematic review identified 24 signatures that were evaluated for TB diagnosis. Seven signatures; Berry86,[25] daCosta2,[40] daCosta3,[40] Francisco2,[41] Kaforou44,[32] Walter47,[27] and Zak16[15] met the minimal TPP for a triage test. However, none of them met the minimal TPP for a diagnostic test in people with other diagnoses.

A prospective cohort study in England evaluated the diagnostic performance of newly derived and published signatures among people suspected of having TB.[42] The best-performing new signature comprising 13 transcripts had an area under the receiver operating characteristic curve (AUC-ROC) of 0·87 with a sensitivity of 77% and specificity of 84%. The best performing published signature had slightly lower performance, with an AUC-ROC of 0·83 (95% CI 0·80−0·86), a sensitivity of 78% (73−83), and a specificity of 76%. Neither achieved the minimal target for diagnostic tests. Similarly, in another prospective cohort study, conducted in South Africa, four signatures (BATF2, Kaforou25, Roe3, Sweeney3) that achieved the highest accuracy did not meet the minimal target for diagnostic tests, with specificities ranging between 86% and 94% at 65% sensitivity.[43] Their performance met the minimal target for a triage test but not the optimal target.

Penn-Nicholson et al discovered a 6-gene transcriptomic signature (RISK6) performing all analyses using qRT-PCR with an aim to translate it into a POC test. In both HIV-infected and uninfected cohorts in South Africa, its diagnostic performance met the minimal target for a triage test. However, in cohorts from Brazil and Peru, the signature achieved the minimal standard only for discriminating active TB from asymptomatic QFT-negative controls but not from QFT-positive controls. However, independent validation of RISK6 by Bayaa et al showed performance met the minimal WHO TPP for discriminating active TB from healthy donors, TB infection and TB treated participants in Bangladesh, Georgia, Lebanon and Madagascar.[44] Penn-Nicholson et al also compared the diagnostic performance using capillary blood against venous blood collected in PAXgene tubes; the diagnostic performance was not significantly different.[16]

A recent head-to-head qRT-PCR comparison of eight parsimonious signatures by Mendelsohn et al showed no signature met the minimal target for a triage test in whole blood specimens from over 3000 HIV-infected or uninfected South African adults, although 5 signatures (RISK11, RISK6, Roe1, Roe3 and Suliman4) met the optimal target in symptomatic HIV-uninfected participants, and four signatures (RISK11, Maertzdorf4, Suliman4, and Thompson5) in symptomatic HIV-infected participants.[45] Whether such signatures have any utility as a triage test only in symptomatic participants remains to be understood.

It is interesting to note that these studies included a sub-set of patients with symptomatic active TB that did not present a typical transcriptomic pattern, characterised by the upregulation of IFN-inducible, complement and myeloid inflammatory gene, leading to imperfect sensitivity of the mRNA signatures.[46] This may be due to misclassification of disease state based on low specificity of symptoms or host-related factors, but it is unclear.

Predicting development of active TB

Zak et al identified a 16 gene signature (later reduced to 11 genes (RISK11, Darboe et al)[47,48] in a cohort of South African adolescents.[15] The signature was capable of predicting TB disease progression up to 18 months before the onset of active TB disease. A criticism of this signature is that it showed somewhat reduced performance in the validation cohort samples from the Gambia suggesting geographic specificity, although this limitation is not restricted to this signature only.

Suliman et al identified a four-gene "Pan African" signature of risk with more generalisable application in South African, Gambian and Ethiopian cohorts of household contacts of TB, although the test set sample size for comparison of these signatures in different nations was small.[28]

Gupta et al evaluated the predictive performance of 17 published signatures using data sets from four studies comprising 1126 samples. The study showed that eight signatures had similar performance to predict incident TB over 2 years with the ROC-AUC ranging from 0·70 to 0·77. However, none of them met the minimal target set for the predictive performance over two years; sensitivities

ranging from 25 to 40% when specificities were optimised at >90%. In contrast, they achieved the minimal target for predicting TB incidence over a short period of 3 months. A recent prospective study similarly showed the prognostic performance of mRNA signatures could meet the target only in the short term.[49]

Recently, a randomised trial assessed the efficacy of TPT guided by transcriptomic signature in South Africa.[50] RISK11-positive HIV-negative participants were randomly assigned into 3-month weekly rifapentine plus isoniazid (3HP), or observation arm (no treatment). While the predictive performance of RISK11 was high, exceeding the optimal target within 6 months, 3HP did not reduce TB incidence over 15 months, although there was some evidence of transient efficacy through 9 months among fully-adherent participants, suggesting more potent therapeutic regimens may be needed in high endemic settings. Additionally, in ambulant people living with HIV, RISK11 identified prevalent TB and predicted risk of progression to incident TB within 15 months.[51] However RISK11's performance approached but did not meet TPP benchmarks for triage and prognostic tests for TB.

Treatment monitoring

mRNA signatures have been shown to decline after treatment of active TB and thus identified as potential markers to monitor treatment response.[11,52] Thompson et al applied Zak16 in a cohort of HIV-negative people with active TB who received treatment (Catalysis Treatment Response Cohort [CTRC]).[53] The signature declined over time during treatment but remained higher than that of healthy controls. The Zak16 signature was able to predict treatment failure (positive culture at month 6) at week 4. They further developed a parsimonious signature (RESPONSE5) comprising a set of 5 genes. RESPONSE5 does not include any IFN-stimulated genes and predicted treatment failure accurately. When combined with Xpert MTB/RIF cycle threshold value, the combination tested at week 4 predicted treatment failure with 83% sensitivity and 97% specificity. Similarly, Penn-Nicolson demonstrated that RISK 6 could discriminate between before, during, and after TB treatment and also showed its high predictive performance for treatment failure (AUC 71·9 at week 1 and 71·5 at week 4).[16] In addition, the signature was validated in cohorts from Brazil, Bangladesh, Georgia, Lebanon and Madagascar, as well as in people living with HIV, whose results did not differ significantly.[44]

Warinske et al evaluated Sweeney3 in the CTRC cohort and its score at the end of treatment successfully identified patients who had failed treatment (AUC, 0·93; 95%CI, 0·83-1·00).[17] Tornheim et al derived 25 genes that were downregulated after 6 months of treatment in Indian children.[54] There was limited overlap with gene lists in other studies. In fact, the performance of the derived signature varied when applied in other published cohorts while the published signatures performed variably in the Indian cohort, highlighting heterogeneous gene expression across different populations.

The use of mRNA signature might enable the individualisation of therapeutic durations and shorten them. In a prospective cohort study, Heyckendorf et al showed that a 22-gene signature (TB22) that can accurately predict relapse-free cure (AUC, 0·94, 95% CI, 0·90−0·98) in people with drug-susceptible TB.[55] When applied in cohorts of MDR-TB patients, it was suggested that treatment guided by the signature could reduce its duration significantly. Currently, large prospective studies including heterogeneous populations are lacking. Moreover, translation into clinical practice would require clinical trials testing treatment strategies guided by signatures.

Diagnostic platform development based on mRNA signatures

Studies described above on the development and validation of signatures mainly used microarrays or RNAseq and a few using in-house PCR methods, none of which are accessible, scalable or reliable for use in high-TB burden countries. Broader use of mRNA-based tests requires a simple test format and quality-assured production at scale.

Cepheid developed an early prototype GeneXpert PCR test that can measure Sweeney3 using whole blood samples[56] called the Xpert MTB Host Response cartridge, A study examined its performance as a triage test or a confirmatory test for active TB in adults living with HIV.[56] When specificity was preset at 90%, its sensitivity was 56% against microbiologically-confirmed TB (positive by either culture or Xpert MTB/RIF) and 86% against Xpert MTB/RIF alone, achieving the minimal target for a triage test. To assess its performance against WHO TPP for diagnostic tests, the authors used a specificity of 95% instead of 98% as considering the limitations of the reference standard. At a specificity near 95%, its sensitivity was 65.7% against microbiologically-confirmed TB, similar to the minimal target of 65%. The same product was subsequently evaluated in a case-control study of TB patients identified through systematic screening in prisons in Brazil and culture-negative controls but did not meet the target for a triage test (a specificity of 53% at 90% sensitivity).[57] Recently, Sutherland et al showed early data that the Xpert MTB Host Response assay, and the single gene GBP5, using fingerprick blood met the minimal TPP for a PoC triage test in blood samples irrespective of geographic area or HIV status.[58] While the sample size was small and only TB symptomatic participants were enrolled, this promising finding demonstrates utility, especially in individuals where a sputum sample may not be available (such as extrapulmonary TB and pediatric TB).

QuantuMDx and bioMérieux are also developing diagnostic platforms using mRNA signatures but no data are publicly available yet.[8]

Comparison with other technologies

There are currently two non-sputum based tests that are endorsed by WHO for diagnosing or screening for TB: LF-LAM and C-reactive protein (CRP). Two studies compared the diagnostic accuracy of Sweeney3 measured using the GeneXpert platform with CRP.[56,57] Both studies suggested better diagnostic performance of Sweeney3 than CRP. In one study, at 90% sensitivity, the specificity of Sweeney3 was 53% compared to 28% of CRP.[57] In the other study, at a specificity near 95%, the sensitivity of Sweeney3 was 66%, significantly higher than that of CRP (14%).[56] We did not find a head-to-head study comparing mRNA signatures with LF-LAM.

Are mRNA based transcriptomic technologies mature enough to develop next-generation diagnostic tests?

Table 2 summarises the findings of the review with reference to the WHO TPP. While a systematic review identified the Sweeney3 signature as a promising marker for diagnosing TB, its performance did not meet the WHO TPP for a diagnostic test in prospective cohort studies.[42,43] There are more promising signatures for a triage test. Whether such tools can be translated into products depend on their ability to meet operational and pricing targets. Sweeney3 has already been evaluated in a PoC platform in people living with HIV, whose performance met the minimal triage target (when compared to Xpert alone).[56,57] Existing mRNA signatures such as RESPONSE5, RISK6, Sweeney3 have also shown the potential to quantitatively monitor disease states. In contrast, there is no promising prognostic signature that can achieve the WHO target over two years. Given the heterogeneous nature of incipient TB, which could progress to active TB at various timings and also regress, mRNA signatures are unlikely to achieve the WHO target. Furthermore, in studies conducted in highly endemic settings, where the force of infection is high, it is difficult to separate the risk of disease from re-activation vs. that of recent re-infection. Short-term prediction is possible but it may largely reflect the detection of subclinical TB. A systematic review by Frascella et al found that >50% of TB was subclinical.[2] In CORTIS, 1% of HIV-uninfected community volunteers had previously undiagnosed microbiologically confirmed TB, with more than 80% being asymptomatic.[50] Such TB cases might need regimens that are different from those used currently used for prevention. We need to define the optimal management guided by tests for short-term prediction.

According to the WHO TPP, the minimal pricing target for triage and diagnostic tests are < US $2 and US$ 6, respectively. Adopting parsimonious signatures is key to reducing costs. The number of genes used in signatures has dropped dramatically over the last decade from 393 to 1-3 genes. However, given the current price of molecular tests (e.g. Xpert XTM/RIF), achieving the targets is challenging in the near future.

Strengths and limitations in existing studies

Study design

Early studies were designed to demonstrate proof of concept and thus were often lacking many elements of studies designed to confirm accuracy with a high level of certainty.[59,60] Studies tended to be small in size and used case-control designs, which can overestimate diagnostic accuracy particularly when healthy individuals were used as a control. A control group should be representative of a clinically relevant population including those with other diseases. Signatures fail to differentiate between TB and other diseases such as pneumonia and acute viral infections that drive expression of IFN stimulated genes (ISGs), the common underlying component of many TB diagnostic signatures for host responses. This was replicated in a recent prospective validation, in which all but the Thompson5 signature that does not contain any ISGs were affected by upper respiratory viral infection.[45] A recent longitudinal study also showed a transient increase in transcriptomic signatures (RISK11) possibly due to viral infections.[61] Other communicable diseases like malaria or COVID-19 and non-communicable diseases like diabetes can affect the performance of host mRNA signatures but this has been insufficiently studied.[62−64] Thus, it is critical that any new signatures are designed in such a way as to allow differentiation of TB from viral and other infections, for example through the exclusion of ISGs and the use of multinominal modelling.[49]

Similarly, risk signatures should include both IGRA-positive and IGRA-negative individuals in the comparator, as it is unlikely that any risk test would perform an IGRA first. The composition of individuals needs to be considered given the prevalence of immune reactivity to *M.tb* in highly endemic areas. Limited coverage of population and geographic regions limits generalizability. The use of samples from different geographic locations to derive a signature rather can increase generalizability.[32] Studies should ideally differentiate performance in HIV-infected vs. uninfected. Recent multi-cohort meta-analysis approach addressed these limitations and three prospective cohort studies have also been published.[42,43,49]

There was wide variation in the number and transcripts that were identified in each study. Several factors including cohort size, diversity of population groups, country-specific studies, diagnostic methods for active TB, sample collection and processing methods employed have been suggested as possible reasons. The resulting variations will have a negative influence by delaying the development and

	Are there signatures that met the performance target?		Have point of care platforms been tested?		Are target costs likely to be met?	
Triage	Yes	• Sweeney 3 and Sambarey 10 achieved the minimal target in an IPD meta-analysis.[17] • BATF2, Kaforou25, Roe3, Sweeney3 achieved the minimal target in a prospective cohort study.[43] • In another prospective study, RISK11, RISK6, Roe1, Roe3 and Suliman4 met the optimal target in symptomatic HIV-uninfected participants, and RISK11, Maertzdorf4, Suliman4, and Thompson5 met the optimal target for PLHIV.[49]	Yes	• Sweeney3 (Prototype GeneXpert PCR): achieved the minimal target when compared to Xpert MTB/RIF as a reference standard (Specificity of 86% at near 90% sensitivity)[56]	No	RT-PCR assays are typically in the range of 10-100 USD even for relatively simple qualitative assays so it would be unlikely that costs for RNA-based tests could be below this; and extremely unlikely that costs could be as low as those required for triage.
Diagnosis	Maybe	• BATF2, Kaforou25, Roe3, Sweeney3 showed specificities ranging between 86% and 94% at 65% sensitivity in a recent prospective study.[43] However, setting a cut-off value that will maintain high specificity across a broad range of settings and patient populations may be a significant challenge and evidence to date is insufficient to demonstrate that this can be overcome.	Yes	• Sweeney3 (Prototype GeneXpert PCR): sensitivity of 66% (at 95% specificity) against a comprehensive microbiological reference standard[56]	Maybe	
Prediction for progression to active TB	Maybe	• Achieved the best performance in an IPD meta-analysis: BATF2, Suliman2, Kaforou25, Gliddon3, Sweeney3, Roe3, Zak16, Suliman4; sensitivities ranged from 25 to 40% over 24 months and 47.1 to 81% over 3 months with corresponding specificities >90%.[18] • In a prospective study, RISK 11 met the optimal target in HIV-negative participants. Roe3 met the minimal target through 12 months. In PLHIV, Roe1 met the minimal target through 15 months.[49]	Maybe	• None tested but Sweeney3 has been tested on the Gene Xpert platform	Yes	

Table 2: **Existing transcriptomic signatures compared with WHO performance and cost targets.**
IPD: Individual participant data; PLHIV: people living with HIV; PCR: polymerase chain reaction.

adoption of a potentially powerful technology for TB diagnostic purposes.

Sample processing

Prior studies have generally focused on venous blood, collected in PAXgene or Tempus RNA blood tubes, which stabilise RNA. There is limited systematic evidence exploring differences between the two methods in terms of yield or RNA quality using commercial kits or automated extraction equipment, although downstream reverse transcriptase steps may be influenced by the fidelity of different enzymes. In addition, the expression of transcripts can differ between venous and capillary blood.[65] Robustness of signatures by the sampling method needs to be evaluated before design lock.

Discovery tools and analysis pipelines

While all the studies surveyed in our review have reported the final set of signature genes, there was very little information on the concentration of the genes or their fold-change. They can impact the choice of the processing steps, detection technology and final sensitivity.

We observed that both microarrays and variants of RT-PCR were used to quantitate expression levels. However, in both cases usually, relative changes in gene expressions are obtained while using a non-standard reference. Reporting formats also vary depending on the platform, and different normalisation methods are often employed. A non-standard reporting model makes it difficult to analytically compare the performance of the different classifiers employed, further contributing to reluctance in adopting microarray-based techniques for diagnostic purposes.

Microarray techniques have been powerful tools for discovery, but are cost-prohibitive and limited by small sample sizes and high false-discovery rates when comparing thousands of potentially differentially expressed genes. Cross applicability and comparison of microarrays to each other and other platforms has proved complex and cumbersome, with strict transformation and normalisation techniques needed to reduce batch effects.

Following the suggestions of Brazma et al[66] who have proposed a Minimum Information About a Microarray Experiment (MIAME) towards standardising microarray data, and Bustin et al[67] who proposed a similar approach for qRT-PCR data, it is recommended that a similar standardised method be adopted in further transcriptomic biomarker studies.

Easily interpretable and applicable models

Models for deriving risk scores are complex and poorly defined methodology hinders independent implementation. Some signatures are published without supporting parameters that may be needed to weight models. This results in signatures that are difficult to interpret and cannot be applied to other settings/ populations in the exact manner as the authors may have intended. These models may also vary by sub-population (e.g. children, PLHIV, etc). For example, the 16 gene risk signature[15] was evaluated as a component of its 16 genes in the Singhania et al study,[46] but not in the signatures intended form as an ensemble of gene pairs. While the authors provided a supplementary excel sheet whereby Ct values for all primers can be uploaded and signature scores provided in output, future models would be well suited to provide open-source scripts in archived databases such as Bitbucket (https://bitbucket.org/).

It is important to validate the performance of signatures using a pre-defined cut-off in prospective studies reflective of clinical practice. However, one cut-off value may lead to differing sensitivity and specificity depending on patient characteristics such as co-morbidities, history of exposure to TB and other diseases, and genetic variation. Thus, the applicability of the cut-off value needs to be validated in diverse populations.

Source of variability

The information of the source of variability is limited. As an example, for IGRA, various sources of variability have been identified.[68] These sources of error were classified as pre-analytical, analytical, post-analytical and manufacturing and immunological. Likewise, it is recommended that such factors be looked into, identified and standardised to reduce variability and improve reproducibility in the diagnostic results from transcriptomic studies.

Deployment in point-of-care platforms

Several fully-automated PoC RT-PCR platforms are now available such as GeneXpert (Cepheid, USA) and Truenat (Molbio Diagnostics, India) that are already endorsed by WHO as sputum-based TB diagnostics[69] with fast followers including the Standard M10 (SD Biosensor, Republic of Korea) and Bioneer IRON-qPCR (Bioneer, Republic of Korea). Through innovations mainly driven by the COVID-19 pandemic, a series of simpler and more affordable near Point of Care PCR platforms have been developed including CovidNudge (DNANudge UK), Accula SARS-CoV-2 Test (Mesa Biotech / Thermo Fisher, USA), ePlexSARS-CoV-2 Test (GenMark Diagnostics, USA), BioMeme SARS-COV-2 (BioMeme, USA) and others, offer opportunities to allow PoC measurement of transcriptomic signatures. To date, none but a few studies have used technologies that can typically be used at PoC.[56]

1. Improve study design by including diverse patient cohorts representing wide geographic areas and conduct an unbiased head-to-head comparison of the multiple signatures using the same assays in the same samples collected from patients with active and incipient TB disease; characterise the impact of other concomitant infections.
2. Standardise methods for specimen collection and processing and create large specimen banks
3. Examine the impact of analytical sources of variability if any
4. Report biomarker abundance (e.g Ct values) and fold-change of gene signatures obtained from a study cohort; include individuals who are unlikely to be infected with TB (e.g. based on tests for TB infection and contact history) as a standard control group
5. Adopt a standardised method for reporting (e.g. MIAME and MIQE)
6. Evaluate the robustness of gene signatures when used with point-of-care technologies
7. Explore and define the optimal management of individuals with positive tests for short-term prediction
8. Conduct validation of design locked products and evaluate their impact on clinical outcomes through clinical trials.

***Box 1*: Recommendations for improved study design, standardisation, and data sharing of transcriptomic research on TB.**

Recommendation and concluding remark

The use of mRNA transcripts has the potential to improve TB diagnostics, prognostics and treatment monitoring substantially. We recommend several actions based on our review (Box 1) to expedite the translation of mRNA-based technologies into global policy and practice. There is a clear need for improved study design, standardisation, and data sharing. Moreover, we need more validation studies of design locked products and, ultimately, their impact on clinical outcomes should be evaluated through clinical trials. We hope that these recommendations facilitate the adoption of a novel PoC diagnostic and contribute to achieving the End TB targets.

Outstanding questions

There are promising signatures for TB diagnosis and triage. Given that existing mRNA signatures are based on the same markers and pathways, more discovery work is unlikely to yield a significant gain in the performance. We need the performance evaluation of mRNA signatures on scalable and affordable products, preferably using capillary blood. Such evaluation needs to be done in diverse populations including those with other diseases.

Existing mRNA signatures are unlikely to achieve targets for long-term prediction. Instead, we need to understand the best treatment regimens in individuals with positive tests for short-term progression.

The performance of mRNA signatures to predict treatment success need to be evaluated in large prospective studies including heterogeneous populations.

Furthermore, the impact of treatment strategies guided by mRNA signatures needs to be tested in clinical trials.

Search strategy and selection criteria

We identified references through a search of PubMed for articles published from inception to May 2021 by using the terms "Transcriptomics," "signatures," "mRNA," and "tuberculosis." We reviewed references identified through the search and references cited in previous articles. We only considered articles published in English. We reviewed identified studies that reported the performance of mRNA signatures as diagnostic tests for TB.

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