Title:

Tuberculosis: Advances and challenges in development of new diagnostics and biomarkers

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ABSTRACT

Tuberculosis remains the top killer from an infectious disease worldwide. Early and accurate diagnosis and detection of drug sensitive and drug resistant tuberculosis is essential for achieving global tuberculosis control. Despite the rollout of the Xpert® MTB/RIF assay as the first-line rapid tuberculosis diagnostic test, the gap between global estimates of incidence and new case notifications remains 4·1 million. More accurate, rapid and cost-effective screening tests are needed to improve case detection. Diagnosis of extrapulmonary tuberculosis, tuberculosis in children, people living with HIV and in pregnant women remains particularly problematic. The diagnostic molecular technology landscape has continued to expand, including the development of tests for resistance to several anti-tuberculosis drugs. Biomarkers to indicate progression from latent infection to clinical disease, to predict risk of re-activation after cure and to provide accurate endpoints for drug and vaccine trials are urgently needed. Sophisticated bioinformatic computational tools and systems biology approaches are being applied to the discovery and validation of biomarkers with substantial progress taking place. New data have been forthcoming through the study of T-cell responses and T cell function, serological studies, flow-cytometric based assays, protein and gene expression studies. Alternative diagnostic strategies include non-sputum based detection with breath-based tests and automated digital radiography under investigation as potential screening and triaging tools. We review recent developments and key achievements in the search for new tuberculosis diagnostics and biomarkers. We highlight current gaps and challenges, and prioritise areas needing further investment, including impact assessment and cost benefit studies.

SEARCH STRATEGY

We searched reports published in English between January 1, 2013 and December 1, 2017 in PubMed, Google Scholar, Cochrane Library, Embase, and websites of tuberculosis related international organisations (WHO, FIND, STOP TB Partnership, TAG, TB Alliance, Consortium for TB biomarkers (CTB2), NIH-NIAID) with the terms "tuberculosis", "*Mycobacterium tuberculosis*", "Latent TB" plus "diagnostics", or "biomarker", "gene expression", "micro-RNA", "proteomics", "metabolomics", "imaging", "interferon gamma release", or "clinical trial". We also reviewed studies cited by articles identified by this search strategy and selected those that we identified as relevant. We also collated and synthesised information on the development of new tuberculosis diagnostics and biomarkers through communications with various stakeholders. Some review articles are cited to provide readers with more details and references than this review can accommodate.

Box 1 Key points

- Tuberculosis is the world's major cause of death from a single infectious disease.
- Rapid and accurate detection of tuberculosis is essential for guiding treatment yet case detection and reporting rates remain low with 40% of estimated incident cases failing to be diagnosed and notified.
- Rollout of the GeneXpert® MTB/RIF assay has not improved global case detection rates and alternative screening and diagnostic tools are needed to assist active case finding that are affordable and suitable for use in settings with few resources.
- A range of technologies are being developed and the majority of rapid diagnostic products close to introduction are based on the detection of mycobacterial nucleic acids.
- Next generation sequencing is improving knowledge of drug resistance mutations.
- There is an urgent requirement for biomarkers that can be used to differentiate tuberculous disease from latent infection, to predict the risk of progression to clinical disease, and to provide accurate endpoints for clinical trials of new drugs and vaccines
- Progress is being made in the search for biomarkers of infection and active tuberculosis disease.
- Obstacles to the production and marketing of new detection platforms are considerable, the foremost being the lack of access to adequate funding for research and development.

INTRODUCTION

During 2016 there were an estimated 10·4 million incident cases and 1·7 million deaths due to tuberculosis.[1] Rapid and accurate detection of tuberculosis is essential for guiding treatment yet case detection and reporting rates remain low with 40% of estimated incident cases failing to be notified. Under-diagnosis remains a problem, particularly in countries where patients face substantial geographic and socioeconomic barriers when accessing health care. In most countries with a high burden of tuberculosis case detection relies on self-reporting of symptomatic patients to a health care facility. Delays in accessing effective treatment provides increased opportunity for transmission and continuation of the epidemic. Detecting extrapulmonary forms of the disease and tuberculosis in children is particularly problematic. Access to tests for drug resistance remains inadequate. In 2016 only 33% of bacteriologically confirmed new (not previously treated) tuberculosis patients were tested for resistance to rifampicin. Of patients that had previously received anti-tuberculosis treatment for at least one month and who are considered at higher risk of resistance 60% were tested.[1] Average treatment success rates during 2016 were 83% but outcomes were considerably poorer for drug resistant disease. In 2014 treatment success rates of 54% and 30% were reported for MDR-TB (resistance to at least isoniazid and rifampicin) and XDR-TB (additional resistance to the fluoroquinolones and second line injectable drugs) respectively.[1]

The WHO-recommended rapid diagnostic test for detection of tuberculosis and rifampicin resistance is an automated polymerase chain reaction (PCR) assay with an integrated semi-automated sample extraction device. The GeneXpert® MTB/RIF assay (Cepheid Inc, Sunnyvale, USA) was endorsed by WHO in 2010. Roll out of the new technology has been facilitated by preferential pricing deals for public sector use in countries with a high tuberculosis burden. However, access remains limited and there are concerns about long term sustainability in countries that are dependent on donor support. New tools for screening and diagnosing tuberculosis are required that are affordable and suitable for use in poorly resourced communities.

There is an urgent requirement for biomarkers that can be used to detect tuberculosis disease and differentiate it from latent infection. In addition, markers that predict the risk of progression to clinical disease would greatly aid efforts to eradicate the disease and indicators of likely treatment failure and relapse would be beneficial for patient monitoring. Biomarkers are also required to provide accurate

endpoints for clinical trials of new drugs and vaccines. The pathology of *Mycobacterium tuberculosis* (MTB) infection and the host response is highly complex and not fully understood. Holistic systems biology approaches are being applied with sophisticated computational and mathematical methodologies. Recently, new data have been forthcoming through study of T-cell responses and T cell function, serological studies, flow-cytometric based assays, protein and gene expression studies. We present an overview of recent developments and key scientific achievements in the search for new tuberculosis diagnostics and biomarkers, summarising the portfolio of rapid diagnostics tests in development and literature pertaining to potential biomarkers as of December 1st 2017. The scientific, operational and resource challenges are also reviewed. Further sources of information and links to relevant WHO polices are listed in the **online appendix 1 (page 1)**.

CURRENT STATUS OF TUBERCULOSIS DIAGNOSTICS

Sputum microscopy to identify MTB acid fast bacilli remains the most commonly performed tuberculosis test. It is a low-cost test that can be performed in basic laboratories attached to primary health care clinics. It has low sensitivity and examination of multiple samples is recommended. Sensitive tests for tuberculosis such as culture and tests for drug resistance have historically been based either in specialist centres or reference laboratories which are not accessible to the bulk of the population.

Figure 1 shows key stages in the development, production and adoption of a new tuberculosis tests. **Online appendix 1 (pages 2 and 3)** provides further details of the technology, target market and links to further information. Concerns regarding the sale of substandard *in vitro* diagnostic assays led the tuberculosis program at the WHO to initiate a process of endorsing tuberculosis tests. Published performance data and information provided by the manufacturers is reviewed by a committee of experts and recommendations are made as to their use. Liquid culture systems and Line Probe Assays (LPAs) for detection of drug resistance were endorsed, but a negative endorsement was declared for serological tests because of their poor sensitivity and specificity. [2]

The Cepheid Xpert® MTB/RIF WHO recommendations for the use of chest X-ray in tuberculosis triaging, screening and diagnosis are provided in (http://apps.who.int/iris/bitstream/10665/252424/1/9789241511506-eng.pdf?ua=1). ® MTB/RIF offers for the first time rapid access to testing for resistance to rifampicin, a marker for MDR-TB. The assay was initially endorsed for use for pulmonary disease in populations with a high prevalence of HIV or MDR-TB

but the recommendation has since been broadened to replace microscopy as a first-line diagnostic and for the detection of some forms of extra pulmonary disease.[3] Although easy to use, the technology is sophisticated and expensive. Reduced pricing is available to the public sector of 145 low and middle-income countries with a high burden of tuberculosis. A GeneXpert machine costs from USD 12,000 to USD 71,000 dependent on the number of test modules incorporated and a single-use test cartridge is USD 9.98 [http://www.stoptb.org/global/awards/tbreach/bet.asp]. The test requires a constant source of electricity and is vulnerable to heat and dust and high rates of instrument failure have been reported in some settings.[4,5] Initial roll-out of the technology was via centralised or reference laboratories. To catalyse uptake at lower levels of the health system from 2013-2016 UNITAID led a project in collaboration with TB REACH, the African Society for Laboratory Medicine (ASLM), Interactive Research and Development (IRD), EXPAND-TB, and the Global Laboratory Initiative (GLI) to make the test more widely available in 21 countries. In total 237 machines and 1.46 million cartridges were provided and 201,748 cases were detected, including 45,278 that were resistant to rifampicin. Unfortunately the project did not monitor treatment initiation and the health impact of the initiative is not known, however the enhanced capacity to detect drug resistance was noted by some countries to have increased awareness of MDR-TB.[5] A new version of the test (Xpert® MTB/RIF Ultra) has recently been launched which is claimed to have increased sensitivity for diagnosis and improved accuracy for detection of rifampicin resistance.[6] A multicentre study reported increased sensitivity particularly among paucibacillary cases, but with a concurrent loss of specificity from 98% to 96%.[7] Detection of rifampicin resistance was the same for both versions of the test (95%). The new Xpert® MTB/RIF Ultra cartridge was endorsed by WHO as a replacement for the Xpert® MTB/RIF cartridge in March 2017.[8] Particularly encouraging is the reported increased capacity to detect tuberculous meningitis where Xpert® Ultra sensitivity was 70% (95%CI 47-87) for probable or definite tuberculous meningitis compared with 43% (95%CI 23-66) for Xpert® MTB/RIF and 43% (95%CI 23-66) for culture.[9]
The Alere Determine LAM TB test is a low-cost rapid lateral flow device for use at the bedside or in a clinic which has been shown to improve survival in hospitalised patients with low CD4 counts when there are limited other bacteriological tests available.[10] It has been endorsed by the WHO.[11]

Screening for latent tuberculosis infection (LTBI) offers opportunity to prevent progression to active disease and interferon release assays (IGRAs) are used to this end in low tuberculosis prevalence settings.[12] However their inability to differentiate LTBI from active disease led the WHO not to endorse their use in countries with a high burden of tuberculosis.[2] There are currently no accurate tests available

for predicting progression to active disease, relapse following treatment or protection following vaccination.

DETECTING DRUG RESISTANCE

MDR-TB and XDR-TB are increasingly reported worldwide. Prompt access to effective treatment is vital to prevent onward transmission and inhibit the emergence of resistance to further drugs during inadequate therapy. Phenotypic culture based methods remain the mainstay of drug susceptibility testing at reference laboratories but they are slow, taking weeks and require stringent microbiology safety precautions. There is no consensus on methodologies, which vary across laboratories and for some drugs the link between microbiological break point and clinical efficacy remains uncertain.[13] In 2017 WHO convened an expert meeting to discuss susceptibility testing for second line drugs and has subsequently recommended changes in the critical concentrations for some drugs.

Drug resistance in MTB is caused by mutations in the bacterial genome that affect drug targets or enabling enzymes. Single nucleotide polymorphisms (SNPs) are the most frequently observed type of mutation. These small changes in the DNA sequence can easily be detected following amplification and they provide a rapid and accurate means of assessing of resistance to rifampicin. Line Probe Assays (LPA), where following PCR of target regions the amplicons are interrogated by membrane-bound probes are available from several manufacturers. LPA are available for rifampicin and some of the other first and second line drugs.[14] There are a number of other tests in development and the pipeline of molecular tests for resistance is summarised in **Table 1**. Three technologies (Cepheid Xpert® Ultra, Genedrive MTB/RIF and the Truenat MTB) have been designed for use at the level of a clinic with microscopy facilities; the remainder are expected to be used in referral laboratories. Gene sequencing is being increasingly used to detect MTB drug resistance as it affords a greater level of accuracy.[13]

The Xpert® Ultra and Line Probe Assays have reported high sensitivities when testing for rifampicin resistance directly from smear positive sputum. Sensitivities are lower for other tuberculosis drugs, due in part to the large number of loci potentially involved in resistance which exceed the testing capacity of these simple molecular devices. A further technology related problem is the recording of false positives when the test is unable to discriminate silent mutations, an example of which is the substitution TTC/TTT in codon 514 of the *rpoB* gene which does not result in resistance to rifampicin.[15] Resistance mutations are not fully characterised for all tuberculosis drugs, and are particularly deficient for the

second line and newer drugs used to treat MDR and XDR-TB. To accelerate progress in this area an international consortium of researchers and international agencies, the 'ReSeqTB initiative' [https://platform.reseqtb.org/] has been established to create an open access platform. Consensus methodology for interpreting the association between mutations and phenotypic drug-resistance has been published.[16]

A new cartridge for the Cepheid GeneXpert is being developed that tests for resistance to isoniazid and some of the second line drugs.[17] A prototype cartridge, when compared to phenotypic tests at sites in China and South Korea, had sensitivities for detecting resistance to isoniazid of 83·3% (95%CI: 77·1,88·5), 88·4% for ofloxacin (95% CI: 80·2 , 94·1), 87·6% for moxifloxacin at a critical concentration of 0·5 µg per ml (95% CI: 79·0, 93·7), 96·2% for moxifloxacin at a critical concentration of 2·0 µg per ml (95% CI: 87·0, 99·5), 71·4% for kanamycin (95% CI: 56·7,83·4), and 70·7% for amikacin (95% CI: 54·5, 83·9). The specificity of the assay was 94·3% or greater for all drugs except moxifloxacin which had a specificity of 84·0% (95% CI: 78·9, 88·3) at a critical concentration of 2·0 µg per ml.[17]

A recent large genome wide association study (GWAS) of MDR and XDR-TB found capacity to detect resistance to ethionamide, pyrazinamide, capreomycin, cycloserine and para-aminosalicylic acid was enhanced by the inclusion of insertions and deletions.[18] This suggests simple SNP detection may not prove adequate for these drugs and more sophisticated molecular devices may be required. Next generation sequencing (NGS) and analysis of the whole genome may eventually become the reference standard for drug resistance identification. Reduced costs and the establishment of high throughput sequencing centres and easy-to-use analytical tools has greatly increased access to NGS and it is currently being implemented as a routine service in several countries. However, in most high burden countries it remains a research tool with the analysis performed overseas. Proof of principle for NGS directly from sputum has been demonstrated but for comprehensive analysis the need to first isolate and culture the organism to obtain sufficient bacterial DNA hinders its application to patient management.[19,20]

TUBERCULOSIS DIAGNOSTICS PIPELINE

Figure 2 depicts the new diagnostics pipeline. There are a number of online resources that track progress in the development of new diagnostics for tuberculosis, including from the Treatment Action Group (TAG)[21] [http://www.pipelinereport.org/2017/tbdx] UNITAID[14][https://unitaid.eu/news-

blog/unitaid-publishes-latest-tb-diagnostic-technology landscape/#en] and a dynamic website established by the Foundation for New Innovative Diagnostics -FIND [https://www.finddx.org/dx-pipeline-status/]. They reveal that most novel technologies reported as 'in development' are not yet close to the market, being either studies of feasibility or early validation of prototype devices.[14] **Figure 3** shows the top ten priorities for diagnostics development. Test developers face considerable technical challenges arising from the pathology of tuberculosis disease. Traditional methods of detecting infectious agents via unique biomarkers have failed for tuberculosis due to the complex and variable host immune response and the paucity of bacteria in clinical samples. Novel approaches being investigated include testing breath for tuberculosis metabolites and the application of nanotechnology and microfluidics to increase detection capacity and reduce hands on sample manipulation. Promising result have been reported on the use of new more sensitive technology to detect LAM [22] and circulating antigen peptides.[23] Those favouring measurement of host response biomarkers will likely require novel platforms that detect multiple proteins. Similarly, antigen detection may require technology capable of assessing molecules of variable size and composition while remaining both easy-to-operate and affordable.

Table 2 lists a range of new tests under development not based on the detection of nucleic acids. Undergoing evaluation for regulatory approval and closest to launch are competitors for the Xpert® MTB/RIF Assay. These are devices aimed at low tech microscopy centres or high throughput instruments with extended drug resistance detection aimed at the reference laboratory. Most tests are sputum-based but some aim to utilize blood or urine which might detect extra-pulmonary tuberculosis and childhood cases who fail to expectorate sputum. Devices are now being designed to run on batteries and aim for a level of robustness that obviates the problems caused by dust and temperature extremes. This includes a portable version of the GeneXpert, The 'Xpert Omni'. However, unforeseen technical challenges have delayed the development of the product and performance data is awaited. To aid prospective test developers and manufacturers a web-based compendium of available resources has been compiled. The TB Diagnostics Pathway [www.tbdxpathway.org] outlines the steps needed for product development from market research and the development of a business plan through to product launch. It includes target technical profiles for tuberculosis diagnostic tools prioritised by the WHO and gives details of specimen banks that can provide samples for validation studies.

Recent advances in communication technologies and cloud-based data management systems have permitted the incorporation of wireless- and cell phone-based reporting within diagnostic devices. This

'connectivity' allows automated reporting of results to the health system and enables monitoring to aid quality assurance measures and stock control.[24] The importance of integration across devices and diseases and the need to maintain confidentiality has spurred the development of national guidelines in many countries that test developers need to be cognisant of when designing connectivity software. Though still in its infancy it is anticipated that the new technology will revolutionise data collection for monitoring tuberculosis incidence and the emergence of drug resistance.

HOST BIOMARKER UPDATES

The measurement of levels of host immune response molecules is a complimentary strategy to the detection of intact MTB or its products. The presence of host markers in accessible samples such as peripheral blood, saliva or urine, would be of great advantage in detecting paucibacillary disease, extrapulmonary disease or where sputum expectoration is problematic, such as in young children. A feature of the adaptive immune response is antigen specificity, where accelerated and enhanced responses follow prior antigen sensitization, allowing association of measured responses with specific MTB antigens. Circulating antibodies against pathogen products are readily measurable in *ex vivo* blood samples by ELISA or similar assays to reflect humoral immune responses, but performance of serological commercial assays has been poor, leading to a WHO advisory against the use of the currently available tests.[25]

Cell-mediated immune responses require stimulation of lymphocytes prior to measurement of changes in the expression of activation markers or effector molecules, like co-stimulatory cell surface molecules or secreted molecules, including cytokines. Such stimulation assays require significant laboratory infrastructure and expertise and take at least several hours to days to produce results. Examples of such include the interferon gamma release assays (IGRAs) and also a range of host marker signatures including host gene expression, protein, metabolic and other host markers. Although there are currently no validated diagnostic tests based on these host markers, recently promising host marker biosignatures have been identified and are in clinical evaluation. Measurement of circulating soluble markers other than antibodies from either the innate (antigen non-specific, rapid response arm of the immune system) or adaptive immune system is also possible in accessible sample types and has the additional advantage of allowing testing without time-consuming stimulation assays. However, the specificity of such measurements could pose obstacles, as there is considerable overlap in responses to many subacute or

chronic insults to the immune system. Interpretation of such measurements will have to be within a well-defined clinical context. One approach to increase disease relatedness is the use of host marker signatures, rather than single markers.[26] Several host signatures are at early stages of development or trial phase as possible new tools for tuberculosis diagnosis (**Table 3**).

Host gene expression

Several studies have explored the utility of host transcriptional biosignatures as diagnostic candidates, as well as biomarkers for prediction of the risk of future development of tuberculosis disease. Genome-wide transcriptional biosignatures detected in whole blood have been the most promising. Some groups have combined transcriptomic and proteomic approaches to explore progression to active tuberculosis disease.[27] A predominantly neutrophil-driven type I Interferon dominated 86-gene whole blood transcriptional MTB signature was reported by Berry et al.[28] The Fc gamma receptor 1B (FCGRIB) gene was found to be highly expressed in tuberculosis patients and in combination with four other genes (CD64, LTF, guanylate binding protein 5 and Granzyme A), discriminated between tuberculosis disease and LTBI with high sensitivity and specificity (94% and 97% respectively) in smaller, case-control studies.[29]

Kaforou and co-workers, investigated blood transcriptional biosignatures in individuals with active tuberculosis, LTBI or other diseases and identified a 44-transcript signature, which distinguished culture confirmed tuberculosis from other diseases with a sensitivity of 100% and specificity of 96% in a case-control study,[30] whereas Laux da Costa et al[31]., building on the findings of the earlier work by Maertzdorf et al, compared the expression profiles of granzyme A, guanylate binding protein 5 and Fc gamma receptor 1A (CD64) genes in blood samples from patients with tuberculosis disease, asthma or non- tuberculosis pneumonia.[31] A combination of the three genes discriminated between active tuberculosis disease and the other conditions with a sensitivity of 93% and specificity of 95%.[31] Sutherland et al. took the work further and evaluated the biomarkers identified by Maertzdorf et al.[41] and other mRNA transcript signatures in 523 study participants from four different African countries using the reverse transcriptase multiplex ligation-dependent probe amplification (RT-MLPA) technique. CD64 was confirmed as a useful marker for tuberculosis disease irrespective of HIV infection and study site.[32] CD64 in combination with three other genes diagnosed tuberculosis disease with a sensitivity of 88% and specificity of 75% in another study.[33] Bloom et al.34 compared the blood genome-wide transcriptional profiles of patients with tuberculosis disease to those of patients with sarcoidosis,

pneumonia, lung cancer and healthy controls in a small study. Although there was significant overlap between transcriptional signatures obtained in tuberculosis and sarcoidosis patients in comparison to other patient groups, 144 transcripts distinguished tuberculosis from other diseases with sensitivity >80% and specificity >90%.[34]

Anderson et al. evaluated mRNA transcript signatures in children with suspected tuberculosis from South Africa, Kenya and Malawi and compared with the profiles of children with LTBI and other diseases.[35] A 51-transcript biosignature, diagnosed culture-confirmed tuberculosis disease in the validation sample set with a sensitivity of 82·9% (95% CI, 68·6 to 94·3) and specificity of 83·6% (98% CI, 74·6 to 92·7). There are multiple studies describing mRNA transcript candidate markers for tuberculosis disease, including *in silico* conducted studies on published data sets and most of the signatures identified in these studies seem promising (accuracy >80%). However, the common limitation amongst these studies is that most of these, even those performed at multiple sites, still employed a case-control design and are at most phase II diagnostic studies.[36] Most of these have small sample sizes and have been at single study sites, due to the high costs involved in properly designed multisite phase III diagnostic studies, including the relatively high costs of RNA sequencing, followed by RT-PCR validation. There is therefore a need to validate the candidate transcript signatures that have so far been identified in prospectively recruited patients with suspected tuberculosis, and in multiple settings.[36] Such approaches would also have to be performed on rapid test platforms that would require minimum training, preferably laboratory-free inexpensive technology, to be of value in high burdened, resource-constrained settings.

A study of South African adolescents by Zak and colleagues, identified a 16 gene transcript signature, which discriminated between adolescents who would subsequently progress or not progress to active tuberculosis in a high incidence setting. The signature had a sensitivity of 66·1% (95% CI: 63·2,68·9) and specificity of 80·6% (95% CI:79·2,82·0), with enhanced sensitivity closer to the time of tuberculosis diagnosis. When evaluated on samples from South African and Gambian household contacts of active tuberculosis cases, the sensitivity of the correlate of risk (CoR) signature was 53·7% (95% CI:42·6,64·3) with a specificity of 82·8% (95% CI:76·7,86), on samples that were collected 12 months before the development of active tuberculosis disease.[26] This performance exceeds the predictive performance of IGRAs or the tuberculin skin test by far. The impact of biomarker-driven preventative treatment of CoR

positive individuals is the subject of a currently ongoing clinical trial [https://clinicaltrials.gov/ct2/show/NCT02735590].

Host protein markers

IGRAs are not useful in high burden settings, owing to the high prevalence of LTBI and the inability of the assays to discriminate between LTBI and active tuberculosis disease. Host markers other than interferon gamma that are produced in response to new or alternative MTB antigens have been investigated. Although multiple antigens and host markers showing potential were identified[37], the performance of tests based on (often overnight) stimulation assays did not warrant the longer lag time to a result and were not suitable for point-of-care rapid diagnostic tests. Yoon et al. reported on a point-of-care CRP finger-prick test as a screening tool for tuberculosis disease in HIV positive individuals with CD4 counts ≤340 cells/ml who were initiating ART in Uganda.[33] This test diagnosed culture-confirmed tuberculosis disease with a sensitivity of 89% and specificity of 72%.[38] However, CRP in combination with six other proteins diagnosed tuberculosis disease in individuals with suspected pulmonary tuberculosis disease in field sites situated in five African countries with a sensitivity of 93·8% and specificity of 73·3%, with CRP as a single maker performing best in HIV infected individuals.[39] A six protein biosignature was also discovered through SOMAscan technology as screening tool for tuberculosis with a sensitivity of 90% and specificity of 80% in over 700 samples from various tuberculosis endemic settings.[40] There are diagnostic tests reported to be in development utilizing various combinations of the reported markers, but data is not yet available on their performance.

Metabolomic markers

Various platforms including nuclear magnetic resonance (NMR) spectroscopy, gas chromatography time of flight mass spectrometry (GC-TOFMS), liquid chromatography high-resolution mass spectrometry (LC-MS), ultra-high-performance liquid chromatography–electrospray ionization-quadrupole time of flight mass spectrometry (UHPLC-ESI-QTOFMS) have been employed to detect small metabolites that can differentiate between tuberculosis disease and other diseases. Although candidate metabolites and pathways have been emerging, the work done so far has been in mostly small, case-control studies and the diagnostic potential of these candidate metabolites is yet to be validated in large studies. Using NMR spectroscopy, Zhou and colleagues evaluated differences in the serum metabolic profiles of patients with tuberculosis disease and healthy controls; building on earlier findings of Weiner et al who identifies

several differentiating metabolites between tuberculosis cases, LTBI cases and healthy controls.[41] They identified 30 metabolites, 17 of which were expressed significantly higher in serum samples from the tuberculosis patients in comparison to controls.[42] They later identified ketone bodies, lactate and pyruvate as metabolites with the most potential in discriminating between tuberculosis disease and other conditions including community acquired pneumonia, diabetes and patients with various malignancies in a follow-up study.[43] In a proof-of-concept study, du Preez et al, employed an untargeted metabolomics approach to investigate host metabolites in sputum samples using two dimensional GC-TOFMS. Although there was high variability in the metabolites identified in samples from different patients, candidate metabolites including fatty acids, mycolic acids and carbohydrates showed potential as biomarkers.[44] Other investigators using LC-MS identified other metabolites including MTB-derived glycolipids and resolvins in plasma samples,[45] urinary metabolites with potential as markers for tuberculosis treatment monitoring,[46] with combinations between four plasma metabolites discriminating between tuberculosis patients and controls including patients with pneumonia (identified by UHPLC-ESI-QTOFMS) with sensitivities ≥ 70% and specificities ≥80%.[47] More metabolic biomarkers are continuously being identified in plasma samples, e.g. metabolites that are involved in the glucose, lipid and amino acid metabolism pathways.[48] Although findings from these studies continue to provide useful information about our understanding of the host metabolic response to infection with MTB, and ultimately help in shedding more light on the intracellular survival of MTB,[49] the potential of these approaches as tuberculosis diagnostics still has to be confirmed. Validated signatures will also have to be translated from the current mass-spectrometry platforms into tools that are appropriate for resource-constrained, high burden settings.

MiRNA

Many investigators have evaluated the role of miRNAs as possible diagnostic biomarkers for tuberculosis disease. Zhang et al evaluated serum miRNA signatures discriminating between active tuberculosis disease, LTBI and healthy controls in a small sample size of 15 tuberculosis patients and 82 controls. They identified different miRNAs that were up or down-regulated in tuberculosis patients (24 and 6 respectively) and although two of these miRNAs (hsa-miR-196b and hsa-miR-376c) showed potential as tuberculosis diagnostic markers after validation by RT-PCR, the limited sample size and lack of a validation cohort limits the global applicability of the study's findings.[50] In a similar study conducted by Latorre et al (blood miRNA signatures in patients with tuberculosis disease, LTBI and healthy controls)

using micro-arrays and validated by RT-PCR, a 3-marker miRNA signature diagnosed active tuberculosis disease (tuberculosis vs LTBI controls) with a sensitivity of 91% and specificity of 88%. [51] In another study, 29 miRNAs were found to be differentially expressed between tuberculosis patients and controls, with three of the miRNAs discriminating between tuberculosis disease and healthy control patients with AUCs between 0·69 and 0·97 after validation by RT-PCR.[52] All of these small, case-control studies require validation in larger cohort studies.

Medical Imaging

Chest X-ray can serve as triaging or screening tool for pulmonary, miliary, pleural and pericardial tuberculosis and can potentially close the case-detection gap when used in appropriate algorithms. It can identify patients in need for bacteriological examination and can provide important information where bacteriological confirmation is unhelpful. It is, however, not widely available in resource-constraint settings. Radiography, which made a major contribution to the eradication of tuberculosis in well-resourced countries of Europe and North America, has become a more feasible option in settings with poor infrastructure with the introduction of digital systems. Software for automated reading is available which provides high throughput with reduced reliance on radiologists and the technology is being used to triage patients during case finding and to assist with prevalence surveys.[14] More sophisticated imaging methods such as computed tomography scanning (CT) offers enhanced sensitivity and fluorodeoxyglucose positron emission tomography/CT (FDG PET/CT) may be used for assessing treatment response, but such specialised instrumentation remains beyond the reach of the majority of tuberculosis patients.[53]

CHALLENGES AND NEEDS

Improved tools for tuberculosis case finding and detection of drug resistance remain top priorities of WHO and the STOP-TB Partnership. Progress is being made but some key research questions for the development of priority diagnostic tests remain to be resolved (see **Table** 4). Validation and evaluation of new technologies is ongoing, with more tests in the pipeline. The ideal test should provide both high sensitivity and specificity but this is not always possible and compromises may have to be made. The accuracy of a new test should be non-inferior to the tests already available but lower sensitivity might be acceptable for technologies that increase case finding due to their suitability for use in communities

with poor access to traditional clinic or laboratory-based health services. High specificity is required if a test is used to initiate anti-tuberculosis therapy but lower specificity can be tolerated when the device is to be used for triaging patients or for community-based screening. Target product profiles published by WHO propose a specificity of 98% for a diagnostic test but a minimum of 70% if a follow-up confirmatory testing will be undertaken.[54] The reported biomarker studies suggest that it may be possible to reach the desired performance targets but for maximum performance multiple markers shall need to be examined. Test developers face two major challenges when developing novel biomarker-based tests. The first is that commercial pressures may prevent selection of the optimum panel of biomarkers. Patenting of biomarkers is common practice and competition to be the first-to-market is a powerful disincentive to cooperation. The second challenge is that of measuring multiple markers across a broad range of concentrations or in very low concentrations. The detection platforms currently used are expensive and unsuitable for use at the point-of-need in countries with a high burden of tuberculosis. When asked how much it would cost to acquire or develop a new detection platform for a novel diagnostic test industry representatives provided estimates of between 3 and 20 million USD.[55] This was in addition to estimated research costs of between 3 and 8 million USD. Thus, although the long term prospects for biomarker-based diagnosis of tuberculosis appears positive these badly needed tests will only be attained if sustained funding is available.

Aside from the biological and technical challenges of creating novel diagnostic devices for tuberculosis there are considerable operational, economic and organisational barriers to be overcome. **Figure 1** presents key stages in production and adoption of a novel tuberculosis test, a process that may take over 10 years and cost in excess of USD 100,000,000. Preparing a new test for market entry may cost more than was spent on developing the device. Studies of test performance must be undertaken in populations of intended use to provide evidence for regulatory approval and WHO endorsement. The lack of a credible gold standard for tests that detect extra-pulmonary forms of tuberculosis is a considerable hindrance. Reliance on sputum based tests is not appropriate and there is a need to increase capacity for diagnosing extra-pulmonary tuberculosis to enable accurate estimates of test accuracy.

Previously there were few diagnostic options for the beleaguered tuberculosis programs to choose from but there will soon be a range of tests and technologies available that appear promising. Shelf life and cost will remain key parameters in decision making but other considerations include accuracy, reliability

and sample throughput capacity. Test performance, predictive value and cost effectiveness may vary by geographic setting or level of the health system due to differing local environmental, demographic and epidemiological factors. There are currently few data comparing test performance and even less on the potential impact and cost benefits. What little data there is often comes from studies involving the developer of the test and one of the greatest challenges currently facing the tuberculosis community is how to fund and organise independent assessment of new technology to maximise the benefits for tuberculosis control.

CONCLUSIONS

Reliance on passive case finding of infectious cases has failed to halt the pandemic and it is now recognised that improved diagnostic technology will not by itself reduce transmission unless interventions are implemented to enable earlier detection and treatment. To eradicate the disease active case finding and screening strategies will be needed and community involvement will be crucial, particularly in the less well-resourced countries. This will require a paradigm shift from national tuberculosis control programs and their supporting donor agencies who have long advocated clinic based detection. In their Tuberculosis Control Report of 2017 the WHO note that insufficient gains have been made and the STOP TB partnership continues to call for increased political commitment and increased funding.

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CONFLICTS OF INTEREST

All authors have ongoing research activities on various aspects of tuberculosis including diagnostics and biomarkers.

AUTHOR CONTRIBUTIONS

Prof Alimuddin Zumla, Dr Ruth McNerney and Prof Gerhard Walzl initiated the idea, developed the first draft outline. Subsequent drafts were developed by Dr Ruth McNerney, Prof Zumla and Prof Tim McHugh, Dr Matthew Bates (diagnostics), Prof Gerhard Walzl, Dr Novel N Chegou and Dr Nelita du Plessi (Biomarkers). All authors contributed to all sections relevant according to their experience and helped finalise the text and content.

LEGENDS TO TABLES AND FIGURES

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Figure 1: **Key stages in the development, production and adoption of a new test for tuberculosis.**

Figure 2: New Tuberculosis Diagnostic Pipeline, August 2017 (courtesy of WHO[1])

Figure 3: Top 10 priorities for tuberculosis diagnostics development

LEGENDS TO TABLES

Table 1: Commercial molecular tests to detect resistance to anti-tuberculosis drugs

Table 2: New diagnostic tests for tuberculosis not based on detection of nucleic acids.

Table 3. Host biomarkers discriminating individuals with tuberculosis disease

Table 4. Current research questions for development of priority tuberculosis diagnostic tests

Figure 1

Key stages in the development, production and adoption of a new test for tuberculosis.

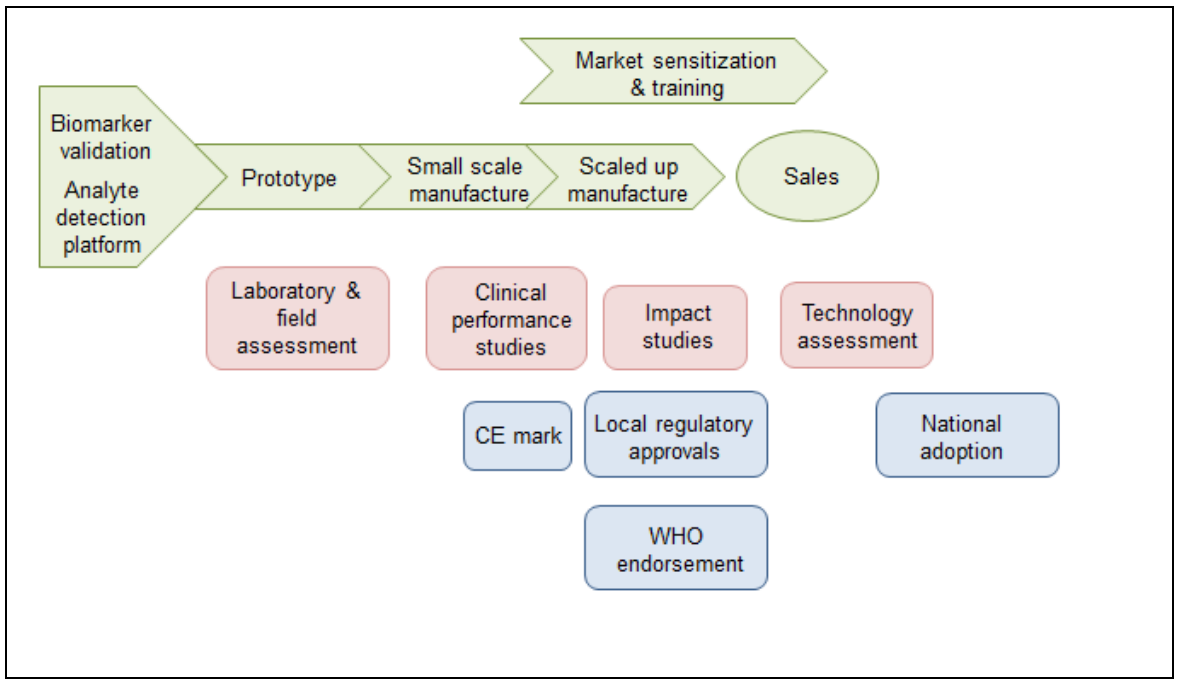

Figure 2 (See editable figure 2 attached)

New Tuberculosis Diagnostic Pipeline, August 2017 (courtesy of WHO[1])

Figure 3

Top 10 priorities for tuberculosis diagnostics development

1. Affordable accurate rapid diagnostic devices for use at the point of care
2. Screening devices for community based case finding or triaging patients in the clinic
3. Non-sputum based tests for sputum scarce patients and extra-pulmonary tuberculosis
4. Validated diagnostic tests for tuberculosis in children
5. Rapid tests for drug resistance to guide the treatment of drug resistant tuberculosis.
6. Tests to predict progression from latent tuberculosis infection (LTBI) to active tuberculosis disease and treatment failure/reactivation
7. Tests to predict vaccine efficacy, to shorten and reduce costs of evaluating new vaccines
8. Independent clinical performance studies to produce evidence for national regulatory approval and WHO endorsement
9. Independent studies to assess impact on health outcomes and on the local health system
10. Technology assessment programs to assess cost benefits, compare technologies and assess diagnostic algorithms.

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