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REVIEW

Tuberculosis Treatment Monitoring and Outcome Measures: New Interest and New Strategies

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SUMMARY Despite the advent of new diagnostics, drugs and regimens, tuberculosis (TB) remains a global public health threat. A significant challenge for TB control efforts has been the monitoring of TB therapy and determination of TB treatment success. Current recommendations for TB treatment monitoring rely on sputum and culture conversion, which have low sensitivity and long turnaround times, present biohazard risk, and are prone to contamination, undermining their usefulness as clinical treatment monitoring tools and for drug development. We review the pipeline of molecular technologies and assays that serve as suitable substitutes for current culture-based readouts for treatment response and outcome with the potential to change TB therapy monitoring and accelerate drug development.

KEYWORDS tuberculosis, treatment monitoring, biomarkers, outcome

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INTRODUCTION

Despite significant progress made in the diagnosis and management of patients in the recent past, tuberculosis (TB) remains a global public health threat with 10 million new cases and 1.5 million deaths annually (1). Recommended TB treatment regimens are long and often associated with severe adverse events, which impact both adherence and therapy outcome (2–5). To monitor treatment progress, clinicians traditionally apply a number of clinical, radiological, and laboratory biomarkers. The World Health Organization (WHO) recommends use of regular sputum microscopy and culture for monitoring of treatment and molecular and phenotypic drug susceptibility testing for identifying the *Mycobacterium tuberculosis* complex (MTBC) drug resistance profile and adjusting the individualized treatment regimen accordingly (3, 6). Although sputum culture conversion (i.e., at month 2 for drug-susceptible TB and at month 6 for drug-resistant TB) has been proposed as a surrogate for treatment outcome (7–9), its use is limited by a long turnaround time, relatively low sensitivity, and high risk of contamination.

Fourteen-day early bactericidal activity (EBA) studies, based on the quantification of TB bacilli in sputum samples and their decline or rise after initiation of treatment, may also be conducted to assess bactericidal activity of antitubercular drugs and their combinations. EBA studies have traditionally relied on the well-established methodology of counting CFU. This approach has several limitations, being both labor-intensive and requiring quality sputum samples with high bacterial burden to provide positive cultures throughout the period of study (10). As an alternative, bactericidal activity may be determined in liquid culture by noting the prolongation of time to positivity (TTP) from baseline (10). This approach gives a more accurate estimate of bacterial burden, but the long time to result limits its use in guiding adaptive trial designs and for clinical management.

TB treatment success is ultimately determined using standardized treatment outcome definitions that are also critically important for the development and testing of novel drugs. The WHO recommends defining TB treatment outcome based on sputum culture positivity and the need for treatment termination or permanent regimen change of at least two anti-TB drugs due to culture reversion, development of drug resistance, or adverse drug reactions (11). However, these definitions do not account for relapse-free survival, which is a more clinically relevant assessment of treatment efficacy (9). To address this, simplified treatment outcomes based on culture conversion and reversion and/or TB relapse within 1 year after treatment completion have been proposed by the TB Network European Trials Group (TBnet) (9). Recently, the WHO proposed updated TB outcome definitions, including an optional definition of sustained treatment success based on the absence of TB relapse within 6 months in case of drug-susceptible and 12 months in case of drug-resistant TB; however, this definition has been designated for operational research use only (12). While all current definitions continue to rely on sputum and culture conversion as monitoring tools, the drawbacks of these methods are recognized, and the need for new treatment monitoring tools is highlighted (12).

Diagnostic technology advances have revolutionized the management of TB. As the most important example, sputum-based, molecular assays, including GeneXpert (Cepheid, Inc., USA), TrueNat (Molbio Diagnostics Private Limited, India), and the line-probe assays (e.g., Hain Lifescience GmbH, Germany), have led to the early detection of drug resistance against important anti-TB drugs (13). Consequently, there is a pipeline of molecular technologies and assays with the potential to change TB therapy monitoring concepts. Such technologies are also potential substitutes for current culture-based readouts for treatment response and outcome. Since phase IIA/IIB clinical studies rely on bacteriological measurements, novel biomarkers may also be potential substitutes for MTBC culture in novel trial designs, accelerating drug development (14). Simpler tools to assess TB therapy response should also be able to signal TB treatment termination (i.e., according to patient response, as opposed to current standard regimen duration). The tests should be both simple and sensitive enough to replace smear microscopy in low-resource settings (15), ideally generating test results within few hours, and

have very high diagnostic performance to predict relapse compared to clinical and microbiological follow-up of patients. Ideally, the test should use nonsputum samples to better ensure operator safety, and result output should preferably be quantitative to allow for better assessment of treatment response over time. The test should be suitable for both TB and extrapulmonary TB patients, including children and those with HIV coinfection.

The aim of this review is to highlight several biomarker candidates with the potential to improve treatment monitoring, accelerate drug development, and ultimately redefine treatment outcome definitions, enabling personalized medicine for TB.

HOST CHARACTERISTICS ASSAYS

Assays measuring the host response to TB infection as a correlate of disease severity or measure of treatment response and cure represent one option to simplify TB treatment monitoring. These approaches include tracking host immune responses through transcriptomic profiling, metabolomic profiling, and monitoring cytokine levels, but also can involve the use of imaging technologies as well as the evaluation of clinical signs and symptoms (Table 1) (16–18). Such technologies are attractive options for treatment monitoring given that they can be performed on readily accessible, nonsputum patient samples (e.g., blood or urine) obtainable in primary health care clinics (PHC) where TB patients often first enter the health care cascade. However, the development of these technologies has generally been challenged given the large range of host variabilities, including infection pathology, host drug metabolism, disease endpoints, coinfections, and even environmental factors (19, 20), as well as the practicalities of translating biomarkers into simple, rapid tests more suitable for PHC. The state and potential of current assays based upon the identification of host biomarkers or characteristics for treatment monitoring are detailed below.

Transcriptomic Profiling

Transcriptomic analyses from whole blood have yielded RNA signatures that correlate with future onset of disease, active TB, and cure (21–24). These signatures often feature genes related to type I interferon signaling (25, 26). Tracking RNA changes in whole blood appears to be a suitable approach for TB therapy monitoring since changes can be found both very early and at later stages of therapy in cohorts, including patients from Africa and Europe with or without HIV coinfection (21, 27–29). However, the potential of these approaches for TB treatment monitoring has yet to be confirmed in larger clinical trials. Still, whole-blood RNA assessment may be used as an alternative surrogate marker for EBA trials since changes appear rapidly following therapy initiation (27). In addition, since RNA alterations can be detected even following culture conversion, RNA may also be a suitable signal for phase IIB study endpoints and ultimately to indicate cure (21, 28). Recently, it was shown that positron emission tomography-computed tomography (PET/CT) changes over the course of TB therapy could be correlated with whole-blood RNA changes (30), though, to date, these changes have not been correlated with relapse in large-scale clinical trials. Furthermore, the potential for secure usage of transcriptomic data involving swarm intelligence to differentiate diseases such as COVID-19 or TB has been presented, supporting this approach (31). Currently, assays for TB treatment monitoring based upon transcriptomic signatures are still in early to middle stages of development, though the pipeline includes blood-based assays suitable for use at various levels of the health care system, from community clinics to district laboratories (32).

Host Adaptive Responses

Cellular immunological markers have promise as candidate nonsputum diagnostics for TB suitable for both adults and children (33). Stimulation of T cells with TB antigens and intracellular cytokine staining specifically measures these markers on TB-specific T cells. While processing of peripheral blood mononuclear cells for this purpose requires specialized capacity and has posed a significant obstacle to the application of this method, a new format for the T cell activation (TAM)-TB assay allows the use of whole

TABLE 1 Examples of assays measuring the host response to TB infection as a correlate of disease severity or a measure of treatment response and cure[a]

Name/type	Host response cartridge	RISK6 score	Transcriptomics panel	TB22	Other RNA signatures	TAM-TB assay	IGRAs
Developer	Cepheid	QuantumDX	Biomerieux	Research Center Borstel	Various	Ludwig-Maximilian University of Munich	Qiagen, Oxford Immunotec
Concept	3-Marker mRNA signature capturing an inflammatory response associated with TB	6-Marker mRNA signature capturing an inflammatory response associated with TB	30-Marker mRNA signature capturing an inflammatory response associated with TB	22-Marker mRNA signature associated with relapse-free cure	RNA signatures with different no. of gene targets involved	Detection of upregulated activation markers on MTB antigen-specific T cells associated with active TB disease	IFN-γ release upon specific stimulation
Use-case	TB detection, prediction, Rx monitoring	TB detection, prediction, Rx monitoring	TB detection, Rx monitoring	TB diagnosis, Rx monitoring	TB diagnosis, Rx monitoring	TB detection in children, Rx monitoring	Diagnosis of latent TB infection
Development stage	RUO, design locked	RUO, design locked	RUO, design locked	In-house	In-house	Design locked	RUO, design locked
Sample	50 μL of capillary blood	50 μL of capillary blood	Tempus tube	PaxGene tube	PaxGene tube	Whole blood, PBMCs	Whole blood, PBMCs
Instrument	GeneXpert 6- or 10-color instrument	Q-POC platform with cassette	BioFire FilmArray platform	Array or RNA-seq	Array or RNA-seq	Fluorescence-activated cell sorting	ELISA, ELISPOT
Time to result	45 min	30 min	120 min	8 h	1–2 days	10 h	1 day
Relevant clinical data	3-Gene signature expression level changed significantly with respect to baseline for 31 pulmonary TB patients who were microbiologically cured by end of Rx. In first month, a median score of 1.97 (IQR = 1.03–2.33) suggested a promising Rx response (150).	Study in patients with and without HIV infection in Africa, Peru and Brazil. RISK6 differentiated between patients with cure and Rx failure (AUC 77.1, 95% CI = 52.9–100) (29).	This assay is currently being validated for diagnosis of active TB in conjunction with risk assessment, clinical context and diagnostic information in South Africa (ClinicalTrialsgov identifier NCT0499540) The Rx monitoring potential of the assay remains unclear.	TB22 model predicted clinical outcomes for TB patients without HIV infection with an AUC of up to 94%, (95% CI = 0.9–0.98) in patients mainly from Europe and Africa (21).	Large list of RNA signatures potentially serving as Rx monitoring tools based upon early exptl data showing Rx monitoring potential (24, 25, 27, 151–153). Certain signatures were identified in TB patients without HIV infection at risk of Rx failure 1–4 wks after start of Rx in South Africa (25, 151).	Frequencies of TB-specific cells from peripheral blood significantly declined over the course of Rx in a cohort without HIV from sub-Saharan Africa (n = 39) with active TB and a 16-yr-old HIV-negative patient with extrapulmonary TB from Afghanistan (33, 154).	A systematic review of 30 studies (24 QFT, 3 T-SPOT.TB, and 3 used both) noted a general decline of IFN-γ over time but with a large variation, concluding that the markers are of no use for Rx monitoring (41).

[a]AI, artificial intelligence; CAD, computer-aided detection; CRP, C-reactive protein; CT, chest tomography; CXR, chest X-ray; DM, diabetes mellitus; ELISA, enzyme-linked immunosorbent assay; IGRA, interferon gamma release assay; LC, liquid chromatography; MS, mass spectrometry; MTB, *Mycobacterium tuberculosis*; NA, not applicable; PBMC, peripheral blood mononuclear cell; PET, positron emission tomography; QFT, QuantiFERON-TB; RUO, research use only; Rx, treatment; TAM, T cell activation marker; TB, tuberculosis.

blood (34). Notably, based on this method, activation markers CD38, HLA-DR, and Ki-67 on TB-specific T cells have been demonstrated to increase at the time of TB diagnosis and to decline with treatment in 10 patients with active, drug-susceptible TB over a 9-month period (35). Ahmed et al. have also noted a slower decline of those markers in HIV-negative patients with active, smear positive pulmonary TB who had a longer time to sustained sputum culture conversion over a 26-week treatment period (with liquid cultures performed at successive time points up to week 26), the primary endpoint used in the PanACEA MAMS TB-01 treatment trial (clinicaltrials.gov NCT01785186), with strong correlation (33). However, these validations of cellular immunological markers were made only against surrogate culture-based endpoints, not against the clinical endpoint of patient cure versus unfavorable outcome. In addition, it was demonstrated that CD27, which appeared to hold promise as a diagnostic marker, did not change with successful treatment in this small (39 patient) cohort. A principal limitation will be that the assay will only work in individuals with a measurable cellular immune response, i.e., a detectable IFN-γ response. However, certain cellular markers have been identified that demonstrate some correlation with baseline disease severity (36, 37), raising the prospect of sputum-independent diagnostic tests that can also inform of disease severity and prognosis, hence aiding treatment decisions and providing a measurement of treatment response in comparison to baseline. These tests, however, currently require facilities for incubation and flow cytometry, limiting their use to research laboratories or other facilities with similar equipment (38). If these assays could be established on the same cytometry platforms used for CD4 testing for HIV care, a wider availability could potentially be achieved. Additional evaluations in trial

TABLE 1 (Continued)

Cytokines	CRP assay	Metabolomics (e.g., tryptophan catabolism)	Deep learning imaging (CAD, CXR)	CT scan	^{18}F-FDG PET-CT	TB scores
Various	Various	Various (LC-MS/MS)	Qure.ai and others	Various	Various	BANDIM
Release of cytokines upon specific stimulation	Quantitative measurement of CRP	Measurement of tryptophan catabolism	AI-based abnormality scoring of paired digital CXR: change in abnormality score is associated with Rx response	Extent of TB lesions on CT scan and change over time	Change of tracer uptake in TB lesions (PET) and lesion change over time (CT)	Baseline and on-Rx clinical markers of risk can predict individual patient Rx success
Diagnosis of latent TB infection, Rx monitoring	Rx monitoring	Rx monitoring	TB detection, Rx monitoring	TB detection, Rx monitoring	TB detection, Rx monitoring	TB detection, Rx monitoring
In-house	RUO, design locked	Experimental, in-house	On market	RUO, design locked	RUO, design locked; additional radiotracers being evaluated	NA
Whole blood, PBMCs	Serum/plasma	Serum/plasma	CXR images and CAD scoring	CT images, trained evaluation	PET-CT images, trained evaluation	None
ELISA	Various	Various	Digital CXR, CAD/AI software	Various	Various	None
1 day	2 h	Days	<1 min	5 min	1 h	Dependent on contributing variables
Several studies have described IFN-γ, TNF-α, IL-4, IL-6, and IP-10 as potential markers for Rx monitoring, though the significantly up-regulated data obtained to date is too heterogeneous to draw a conclusion that these cytokines are useful for Rx monitoring (42–45).	This assay has shown potential for Rx monitoring in active TB patients (with and without HIV infection). One study of HIV-positive and -negative patients in South Africa showed CRP decline to ≤55% of the baseline value by wk 2, a prediction of hospitalization or death with 99% negative predictive value was described (44–46).	A study in samples from 48 TB patients, 20 TB-DM patients, and 48 healthy controls without HIV infection from Indonesia revealed a three-target model to distinguish between groups (AUC = 0.91–0.97) and identified metabolites that lowered during Rx. Another study conducted in samples from HIV-negative, pulmonary TB patients from Georgia focused on decreased tryptophan/kynurenine ratios, which changed under Rx, and identified indoleamine 2,3-dioxygenase-1 as a potential target for host-directed therapy (52, 53).	Deep learning software has mainly been applied to CXRs from active TB patients at baseline for TB detection (64, 155, 156). Its value as Rx motoring tool is not yet well described.	CT findings correlate with Rx outcome, with larger cavities identified during Rx having a high predictive value for Rx failure in an HIV-uninfected South African drug-susceptible TB cohort. Cavity wall thickness and the total volume of intraparenchymal radio-dense lesions at the end of Rx were also found to have high predictive value of Rx failure in this cohort (65–67).	The potential of PET-CT scans to serve as markers for Rx shortening is currently under evaluation in a randomized control trial. PET-CT has correlated with clinical and sputum markers in HIV-negative TB patients in an EBA trial in South Africa (NCT02821832) (79, 80).	Several Rx response scores have been presented involving clinical, microbiological and radiological data. A risk stratification tool using patient demographics and clinical parameters (from phase III trials) in drug-susceptible, HIV-negative TB patients identified groups at low, moderate, and high risk for poor outcome in Germany (67, 85–88, 157).

settings will be needed to confirm the clinical relevance of these markers for TB treatment monitoring.

Cytokines and Other Biomarkers

Proinflammatory cytokines produced by TB-specific T cells are well established as significant contributors to TB immunity (39, 40), as demonstrated by the inability of the host to control infection in their absence. The importance of IFN-γ expression in TB-infected subjects is reflected in the assay used to determine infection in the first place: commercially available IFN-γ release assays (IGRAs) such as QuantiFERON TB Gold (Qiagen, Germany) and T-SPOT-TB (Oxford Immunotec, Ltd., UK) assays frequently replace tuberculin skin tests (TSTs) to identify patients infected with TB. While levels of IFN-γ production in IGRAs have been suggested as a potential biomarker for treatment monitoring, a systematic review of 30 studies did not find uniform patterns of IGRA levels following treatment due to the high degree of variation between participants, undermining the usefulness of these tests for treatment monitoring (41). A more promising approach may be to directly measure cytokines circulating in plasma or serum samples, removing the added variability inherent to *in vitro* stimulation. Several technologies are available for this purpose, including simple enzyme-linked immunosorbent assays (ELISAs), as well as multiplexed bead-based and plate-based approaches (42–44). Using these approaches, it was demonstrated that several cytokines, including IFN-γ (42), TNF-α (42), IL-4 (42), IL-6 (44), and IP-10 (45), were increased in active TB disease compared to healthy or latently infected individuals, confirming their association with disease severity, and declined with TB treatment. These studies included evaluations of protein levels in blood samples from 319 active TB, HIV-positive and -negative patients at baseline versus following 8 weeks of combination therapy in TBTC Study 29 (44), plasma samples from 42 HIV-positive and -negative individuals with active pulmonary TB undergoing 26 weeks of treatment followed over 18 months in Durban, South

Africa (45), and 67 patients with drug-susceptible TB disease followed over 6 months of treatment in Beijing, China (42). Unfortunately, even though multiple studies have measured the same cytokines, the evidence base is heterogeneous, suggesting poor sensitivity and specificity of these markers for the prediction of treatment progress. To date, no cytokine-based assay for treatment monitoring has progressed from early development stages, and currently used technologies are limited to central laboratories.

Beside cytokines, new or updated proteomic approaches have identified additional serum proteins as potential biomarkers for anti-TB treatment monitoring. Commercially available, C-reactive protein assays based upon finger-prick blood testing have already undergone WHO review and recommendation for community triage in people living with HIV (32) and the C-reactive protein has additionally been shown to consistently decline during treatment (44, 46, 47). In an assessment of 33 patients in a high-TB and -HIV setting with serial changes in C-reactive protein evaluated over 8 weeks (46), the failure of C-reactive protein to reduce to $\leq$55% of the baseline value by week 2 predicted hospitalization or death with 99% negative predictive value for patients with confirmed or presumed TB. Apolipoproteins and members of the complement cascade are also modulated by TB treatment and have been suggested as components of predictive proteomic signatures (48–50).

In addition, one study with access to patients who relapsed following treatment completion assessed whether a proteomic signature would be able to predict treatment outcome (51). Ronacher et al. showed distinct patterns of changes in immune markers (including cytokines, chemokines, soluble receptors and acute phase proteins) following treatment initiation in 78 cured and 12 relapsed HIV-negative patients with drug-susceptible TB from the Action TB Study who were used as a discovery cohort. A combination of four immune markers (TNF-β, sIL-6R, IL-12p40, and IP-10), in addition to TTP and body mass index at diagnosis, was able to discriminate relapsed from cured patients with an Area Under the Receiver Operating Characteristics (AUROC) curve of 0.819 (95% confidence interval [CI] = 0.679 to 0.942) in this discovery cohort, and this was validated in a second cohort of 23 cured and 17 relapse patients from Uganda and Brazil with an AUC of 0.718 (95% CI = 0.509 to 0.903). Interestingly, the predictive potential of this biosignature was most pronounced when measured at baseline since immune markers tended to normalize during treatment (51). As multiplex assays, such as those of SomaLogic, Inc. (USA), and O-link Holdings AB (Sweden), are becoming more widely available, confirmatory studies will allow for a targeted assessment of some of these markers as potential predictors of treatment outcome.

Metabolomic approaches are similarly being assessed for their potential for anti-TB treatment monitoring. Multiple metabolic pathways are affected by TB infection, with tryptophan catabolism in particular being heavily influenced by TB and its treatment (52, 53). Of note, this metabolic pathway is also being explored as a target of host-directed therapies, with blockade of indoleamine 2,3-dioxygenase (IDO; the rate-limiting host enzyme for catabolism of tryptophan to kynurenine) activity showing promising results in reducing clinical manifestations and pathological correlates of TB in a macaque model (54). Further studies will be necessary to determine whether these early findings translate clinically for TB patient treatment monitoring.

Imaging

Chest X-rays (CXRs) are a fast and inexpensive way to screen for active TB with many portable devices now available for testing in community settings. However, the process is subject to interpretation and thus the accuracy of this method largely depends on the experience of the reader. The use of computer-aided detection (CAD) technologies for TB detection in digital CXRs has the potential to standardize interpretation and improve the feasibility of CXRs for widespread TB screening and diagnosis (55). CAD software generates a score from 0 to 100 with some products, such as qXR from Qure.ai (India), further allowing comparisons of images obtained during treatment to provide a quantitative measure of treatment success (56). Recently, the WHO recommended three specific commercially available CAD software packages after

finding the diagnostic accuracy and the overall performance of the software to be similar to the interpretation of digital CXR by a human reader (57). Many studies have additionally demonstrated the potential for CXR to be used for monitoring bacterial load and estimating TB disease severity alone and in combination with smear microscopy (58–61), with evidence of the association between extensive parenchymal involvement and 2-month culture conversion, as well as the number of cavities with relapse in non-HIV-infected patients (62). However, despite strong associations with patient-important treatment outcomes (58), studies have had conflicting results, as an assessment of the potential for treatment shortening in patients with noncavitary TB (with culture and CXR) found that month 2 sputum culture conversion and the absence of cavitation was insufficient to support treatment shortening (63). Ultimately, this use case has not been well evaluated for CAD software to date, with most studies using human readers (62, 63), and the potential of this technology for treatment monitoring remains questionable (64).

Chest computed tomography (CT) is more sensitive and specific for active TB diagnosis than CXR given cross-sectional imaging and higher resolution, and changes in CT findings over time have been shown to correlate with treatment outcome (65). Notation of greater volume of cavities on chest CT performed at baseline, month 1, and month 6 of treatment had a high predictive value for treatment failure in an HIV-uninfected South African drug-susceptible TB cohort (66), consistent with evidence that the presence and extent of cavities at initiation and conclusion of TB treatment is a risk factor for poor treatment outcome or delayed culture conversion (67–69). CT-detected cavity wall thickness and the total volume of intraparenchymal radiodense lesions at the end of treatment were also found to have high predictive value of treatment failure in the same cohort (66). However, only eight patients had treatment failure in this specific cohort, and it is notable that a significant proportion of individuals continue to have residual chest CT abnormalities even after apparent successful completion of TB treatment. It can be difficult to determine the presence of live TB bacteria in these lesions and the risk for subsequent disease recurrence with CT alone (70, 71). Combining ^{18}F-fluorodeoxyglucose (^{18}F-FDG) positron emission tomography (PET) with CT imaging (PET/CT) is an area of great interest for TB treatment monitoring to address this limitation. ^{18}F-FDG accumulates in metabolically active cells, including inflammatory cells typically involved in active TB lesions (i.e., neutrophils, macrophages, and lymphocytes) (72). PET/CT thus overlays functional information about metabolic activity onto the pathological lesions identified on CT. Data from multiple cohorts have demonstrated that PET glycolytic activity decreases in response to effective TB treatment and can predict treatment outcome (31, 66, 73–78), though persistent lesions with ongoing inflammation, as well as new inflammatory lesions after completion of successful TB treatment, have also been noted (75, 76). The significance of these heterogenous patterns is unclear. The ability of PET/CT, along with other clinical and microbiologic parameters, to identify individuals with drug-susceptible TB for whom first-line anti-TB treatment can be shortened to 4 months is currently being tested in a randomized trial (NCT02821832) (79). PET/CT is also being explored for its potential role in identifying new drugs and regimens in EBA trials through the information it provides on lesion volume and PET glycolytic activity (80). The high cost and logistical complexity of performing PET/CT limit its broader implementation as a treatment monitoring tool, but this biomarker nonetheless holds promise as a potentially useful translational tool for confirming preclinical results of promising new drugs and regimens in earlier stage phase I/II trials before undertaking large expensive phase III trials.

Clinical Score, Signs, and Lung Function

A range of risk factors have been shown to be associated with poor TB patient outcomes, including demographic variables such as age, sex, ethnic background, and occupation, and clinical variables such as clinical form of TB, history of TB treatment, disease severity (e.g., smear grade and cavitary disease), comorbidities (e.g., HIV coinfection and malnutrition), and clinical signs and symptoms (e.g., weight, cough, lung

function, and chest pain) (67, 81–84). Although current practice guidelines suggest that certain risk factors may indicate the need for adjustment of treatment regimens (e.g., treatment duration) (82), clinical risk scores and risk stratification algorithms using a suite of readily available predictors can be useful to further differentiate individuals to monitor treatment response, in both drug-susceptible and drug-resistant TB populations (67, 85–88). For example, a risk stratification tool has been developed using patient demographics and clinical parameters from pooled data obtained in contemporary phase III trials in drug-susceptible TB-treated patients, leading to identifying groups of low, moderate, and high risk for poor outcome (67, 89). In a patient population where the high-risk group had unfavorable outcome rates of roughly 30%, the tool provided support for the use of clinical scoring systems and algorithms to triage patients at baseline or during treatment to improve the probability of treatment success. Although clinical scoring systems and algorithms may provide a simple approach to evaluate response to treatment, careful consideration is required on the mechanisms for handling of missing data, on the cost, resources, and devices/tools necessary to measure variables, and on the high interobserver variability for subjective measurements (e.g., clinical symptoms). Importantly, more research is needed to assess the utility of clinical scoring systems to predict treatment outcomes and monitor treatment response across regimens of various compositions and dosing, as well as by demographics (e.g., ethnicity and sex) and clinical factors (e.g., weight and HIV status) that have also been associated with poor outcomes (68, 90–92). The overall moderate performance of clinical scoring systems may benefit from combination with drug exposure variables, along with other more quantitative and sensitive bacterial or host-derived markers, to serve as integrated tools to maximize precision in predicting treatment outcomes.

PATHOGEN BURDEN AND FITNESS ASSAYS

Treatment monitoring tools based upon the quantification of viable bacteria allow for better precision in TB clinical care by providing a specific metric for TB treatment efficacy and outcome prediction (93). Although TB burden may be inferred by growth-based MGIT culture, this method is time-consuming, requires sophisticated laboratory infrastructure, and is prone to contamination. While molecular methods to estimate TB burden such as Xpert MTB/RIF (Cepheid, Inc.) can overcome these limitations, the presence of DNA from dead or killed bacilli, which can remain detectable for months and even years after treatment, limits the use and interpretation of findings (94, 95). In this context, rapid and simple molecular assays to quantify both MTBC burden and viability in a patient sample present useful options for TB treatment monitoring (Table 2).

Sputum-Based Molecular Load Assays

The TB molecular bacterial load assay (TB-MBLA) is a culture-free assay that specifically detects and quantifies the viable MTBC bacillary load from a patient's sample via reverse transcriptase quantitative PCR (RT-qPCR) in $<$4 h (96). By detecting 16S rRNA (rRNA), a component of the multiple ribosomes in a viable cell, the test can detect as few as 10 to 100 bacteria in a milliliter of sample (97–101). Targeting RNA makes TB-MBLA an accurate measure of live bacteria and distinguishes it from molecular tests that detect DNA (99, 100) and differentiates the assay from culture-based methods of monitoring the response to anti-TB therapy, where the TTP increases as patients clear their bacterial load (100). As early as 3 days after the initiation of therapy, TB-MBLA effectively detects the bactericidal effect of anti-TB therapy and is able to provide long-term assessment of slow treatment responders (100, 102). The assay has the added advantage of detecting viable, nonculturable MTBC bacilli, obviating complicating factors of media variability and dormant cell states (103, 104), as well as the need to decontaminate sputum, removing a significant variable in laboratory processing (99). It is also possible to perform the assay following heat inactivation of sputum samples with a small, but predictable, loss in measured viable count (105), reducing the need for expensive, high-containment facilities. A design locked TB-MBLA is entering

TABLE 2 Examples of assays based upon the quantification of viable *M. tuberculosis* bacteria to monitor treatment response and predict outcome[a]

Name/type	Solid and liquid culture	Xpert MTB/RIF ultra	PMA/eMA pCR	TB LAM assays	TB-MBLA	Culture-free TB test	rRNA synthesis ratio	tNGS (e.g., Deeplex Myc-TB)	WGS
Developer	BD and others	Cepheid	Various	Otsuka, LSI Medience Corporation, FujiFilm Corp., SD Biosensor, Inc.	LifeArc	Tauns	University of Colorado	Genoscreen	Illumina and others
Concept	Time to positivity in liquid culture/count of CFU on solid culture	Semiquantitative detection of mycobacterial DNA. Use of Xpert cycle threshold for estimate of bacterial burden and treatment monitoring	Membrane-permeable dyes modify and prevent amplification of dead cell-derived DNA, allowing quantification of viable bacteria via PCR or RT-PCR	Quantification of LAM in sputum as a correlate for MTBC burden	Quantification of rRNA as a correlate with the burden of live bacilli	Quantification of MPT64 released from live MTBC following 1 h of heat treatment of the sample correlating with MTBC burden	Quantification of the ratio of rRNA and liable splice as a correlate of bacterial burden and fitness	tNGS targets genes associated with TB drug resistance and identifies lineage	Genome sequencing provides complete mutation information for MTBC, including identification of TB drug resistance mutation and MTBC lineage
Use-case	EBA, Rx monitoring	Diagnosis of TB, Rx monitoring	EBA, Rx monitoring	EBA, Rx monitoring	EBA, Rx monitoring	EBA, Rx monitoring	EBA, Rx monitoring	Differentiating new infection/relapse; identification of resistance and resistant populations	Differentiating new infection/relapse, Identification of resistance and resistant populations
Development stage	On market	On market	In-house	RUO	RUO, on market	CE-MARK	In-house	CE-MARK	In-house
Sample	Sputum	Sputum	Sputum	Sputum and urine	Sputum	Sputum or other	Sputum	Sputum	Sputum
Instrument	MGIT	6- and 10-color GeneXpert System	In-house PCR	Various ELISA and CLEIA and associated platforms (e.g., PATHFAST)	PCR	Automated ELISA	PCR	Illumina, Nanopore	Illumina, Nanopore
Time to result	Up to 8 wks	2 h	4–6 h	Unknown	<4 h	2.5–3 h	Unknown	48 h	48 h
Relevant clinical data	Time to culture conversion, as well as 2- and 6-month culture conversion status, are currently used as surrogates for Rx response and outcome. The number of CFU on solid media or the time to positivity are also used to quantify bacteria in EBA studies (8, 9, 158–160).	Xpert Ultra appeared to be of no use as Rx monitoring tool in HIV-positive and -negative TB patients from the REMOX trial ($n = 221$, Cape Town, South Africa, and Mbeya, Tanzania). Furthermore, Xpert results had a high sensitivity but very low specificity when smear and culture were used as reference standard (sensitivity (97.0%, 95% CI = 95.8−97.9), specificity (48.6%, 95% CI = 45.0−52.2), though results are conflicting and the use of cycle threshold for treatment monitoring is still being explored (161–163).	The method seems to be promising to specifically detect viable MTBC bacilli, with a specificity of 84.6% and a sensitivity of 84.6% compared to counting CFU. No clinical evaluation has been performed to evaluate the potential of this technology for Rx monitoring to date (107–109).	A study involving 57 TB patients showed that HIV-positive and -negative participants with positive LAM test at month 2 had a 5.6-fold (95% CI = 1.2 to 25.2) greater risk of mortality. Another study involving 40 drug-susceptible pulmonary TB patients demonstrated that decline of sputum LAM concentrations during the first 56 days of therapy correlated with increases in time to culture positivity, with notable changes during the first 14 days (120, 121).	The MBLA corresponded well with culture in several early-phase clinical studies. In a study involving samples from 178 HIV-positive and -negative patients from Southeast Africa, it was shown that individuals with high pretreatment bacillary burdens were less likely to convert to negative by wk 8 of Rx than those with a low burden (96, 97, 100).	A study conducted in Taiwan involving 1102 patients with suspected TB infection revealed a sensitivity of 86.9% and a specificity of 92.0% with culture as reference standard. A follow-up study with HIV-negative TB patients from Japan showed a specificity of 89.5% to predict negative culture results on day 14 (114, 115).	The rRNA synthesis ratio was quantified for sputa from 17 Ugandan, 28 Vietnamese, and 19 Beninese HIV-positive and -negative patients treated with HRZE for drug-susceptible pulmonary TB. Rx was demonstrated to lead to a rapid decline of the rRNA synthesis ratio (106).	tNGS has been demonstrated to rapidly provide clinical data to guide personalized Rx and Rx management, with the potential for detection of acquired drug resistance, including low-level resistant populations, during Rx (140, 164).	WGS is the method currently used to fully, genotypically characterize MTB, allowing for differentiation between reinfection and relapse in principle. WGS, like tNGS, can also guide personalized Rx and Rx management with the potential for detection of acquired drug resistance, including heteroresistance (137, 165).

[a]CLEIA, chemiluminescence enzyme immunoassay; EBA, early bactericidal activity; ELISA, enzyme-linked immunosorbent assay; EMA, ethidium monoazide; LAM, lipoarabinomannan; MBLA, molecular bacterial load assay; MPT64, *M. tuberculosis* complex protein 64; MTBC, *Mycobacterium tuberculosis* complex; PMA, propidium monoazide; RT-PCR, real-time PCR; RUO, research use only; Rx, treatment; TB, tuberculosis; tNGS, targeted next-generation sequencing; WGS, whole-genome sequencing.

clinical evaluation with the hope that, if successful, it can be introduced into the clinic as a suitable substitute for culture conversion for monitoring treatment response.

The mycobacterial precursor rRNA synthesis ratio assay has additionally shown promise in quantifying the ability of various anti-TB treatment regimens to potentially shorten treatment (106). This assay operates on the principle that rRNA synthesis in MTBC is distinctly impacted by sterilizing versus nonsterilizing drugs. Sterilizing drugs and highly effective drug regimens profoundly suppress *M. tuberculosis* rRNA synthesis, whereas nonsterilizing drugs and weaker regimens do not. Thus, the rRNA synthesis ratio provides a metric for drug effect and bactericidal activity that may help to differentiate sterilizing drugs and regimens that shorten treatment and promote cure (i.e., suppressing MTBC rRNA synthesis), as opposed to drugs and regimens that allow infecting MTBC to maintain rRNA synthesis. Although this technology is only recently being developed for TB treatment monitoring, assays including quantification of rRNA synthesis to this end may serve as a marker of the ability of a drug or drug regimen to shorten TB treatment.

Another sputum-based option to discriminate viable bacilli is to use propidium monoazide (PMA)- or ethidium monoazide (EMA)-PCR or real-time PCR. The PMA and EMA dyes can penetrate the bacterial membrane, where they modify DNA derived from dead cells, preventing PCR amplification. These assays have been demonstrated to rapidly discriminate dead from viable *M. tuberculosis* cells in early studies (107–109), avoiding the limitations of PCR-based assays such as Xpert MTB/RIF and Xpert MTB/RIF Ultra (Cepheid, Inc.), which are capable of detecting DNA from dead bacteria (94, 95), though further clinical evaluations will be necessary to determine the potential of PMA/EMA assays for anti-TB treatment monitoring and outcome.

The shorter time to result and the absence of the need to decontaminate samples mean that RNA-based assays like the TB-MBLA and rRNA synthesis ratio assays and DNA based assays such as PMA/EMA have the potential to inform clinical decision-making in real time (100). Furthermore, the decline in bacillary load correlates with patient resolution of clinical signs, particularly cough, and differentiates the patient response to different regimens (100, 110), which widens the application of these approaches to clinical trial monitoring.

M. tuberculosis Complex Protein 64 Release Assays

Studies suggest that the load and fitness of bacteria in a sample can be quantified by providing nutrients to it and measuring the induced protein expression response (111), by incorporating fluorescent trehalose in the *M. tuberculosis* membrane (112), or by conducting short-term culture with resuscitation promoting factors (113). Another recently described approach is to briefly expose the patient sample to 46°C heat to specifically induce the release of *M. tuberculosis* complex protein 64 (MPT64) from live bacilli (114). The MPT64 release assay has demonstrated 88% sensitivity and 97% specificity for TB detection, with MPT64 release strongly correlating with both sputum smear grade and MGIT TTP (115). When measured in sequentially collected sputum samples (days 0, 14, and 28) from 50 active pulmonary TB patients undergoing treatment, MPT64 release was strongly correlated with treatment response, with a sensitivity of 81% (95% CI = 58.1 to 94.6) for predicting positive day 28 cultures (116). The MPT64 release assay is currently under early development by Tauns Laboratories, Inc. (Japan), and in particular, given the simplicity and speed of the test, this approach could provide a useful alternative to the time- and labor-demanding phenotypic methods currently used for treatment monitoring, though it will likely still be limited to central laboratories, and it has been demonstrated to have some lineage-specific limitations (i.e., MPT64 is a poor predictor for L5 MTBC strains) (116). In addition, there are some concerns that MTBC genetic polymorphisms in the *mpt64* gene change the MPT64 antigen sufficiently to result in false-negative results for MPT64 detection assays (117).

Lipoarabinomannan

Lipoarabinomannan (LAM) is a prominent glycolipid in the cell wall of *M. tuberculosis* with utility as a pathogen biomarker to assess patient therapeutic response (118, 119). As

demonstrated by Drain et al., LAM decreases during anti-TB therapy, and patients with detectable LAM after an intensive phase of therapy appear to have greater mortality risk (120). As a marker of active TB, LAM is an attractive target for TB diagnostics and treatment monitoring, including one assay measuring LAM in sputum developed by LSI Medience Corporation (Japan) for EBA studies and treatment monitoring (121), and a number of urine-based LAM diagnostics, e.g., the SILVAMP TB test by FujiFilm Corporation (Japan) and the TB LAM assay by SD Biosensor, Inc. (Republic of Korea) (122, 123). Compared to sputum, urine samples are easily obtained from patients, have shown no evidence of TB transmission, and provide an opportunity to better diagnose TB in patients who have trouble producing sputum, such as HIV patients, children, and patients with extrapulmonary TB (120). Urine-based lateral-flow tests are also rapid, and there is no need for sophisticated laboratory infrastructure or highly trained technicians, making these technologies appropriate for use in community settings. However, there is generally less LAM present in urine compared to sputum (124), and the performance of LAM-based assays can be highly variable (125, 126), although their general performance appears to be at least as good as smear (121). In addition to urine-based LAM, serum-based LAM detection presents another option for treatment monitoring. However, even less LAM is present in serum compared to urine, and therefore lower detection limits of serum-based LAM diagnostics must be achieved to ensure these technologies are sufficiently sensitive to be useful as treatment monitoring tools. A number of LAM-based assays are at various stages of development, though to date only the urine-based LAM assays have demonstrated adequate diagnostic accuracy while maintaining ease of use and rapid time to result (121). Generally, these assays have shown higher sensitivity for HIV-positive compared to HIV-negative subgroups, with diagnostic performance correlating with immunosuppression (127). Clinical studies will be key to determine the potential of these assays for anti-TB treatment monitoring in all patient populations.

Whole-Genome Sequencing, Strains, and Fitness

MTBC has been considered to be of clonal origin due to the low level of overall genomic variation (128). However, very little consideration has been given to the fact that lineages could influence the clinical picture of the disease as well as drug response. Emerging data now associate specific lineages with clinical manifestations, including increased virulence of infections and the capacity to acquire drug resistance (129–133), directly impacting treatment response. In addition, some lineages may have an increased MIC to new anti-TB drugs and may require both an adjustment of the dosage and the critical concentration to be tested "*in vitro*" to inform treatment regimen selection (134, 135). For these reasons, it is crucial that any new trial for drugs and diagnostics includes settings where different lineages are sufficiently represented and drug effects are considered in light of MTBC lineage and sublineage.

Whole-genome sequencing (WGS) not only provides important information regarding MTBC lineage but also serves to screen for drug resistance development during treatment (136). Although WGS can be performed directly from smear-positive samples (137), it is mainly performed on isolates grown in culture, even though early liquid cultures are suitable specimens to lower the time to result. Targeted next-generation sequencing (tNGS), which detects known and novel variants in specific gene regions as opposed to the entire genome, is another attractive option for use in primary samples for similar purposes, with the advantage of detecting the presence of multiple strains in a primary specimen (i.e., mixed infection) and allowing the detection of minority variants bearing mutations associated with drug resistance (138). Since there is evidence that even slightly elevated MICs to first-line drug compounds can impact treatment outcome (139), tNGS presents a distinct advantage to identify resistance markers even when present in minority populations. In addition, the use of tNGS/WGS on smear-positive samples during therapy is the most efficient tool to promptly identify emergence of resistance. In addition, sequencing is the only tool currently capable of providing genomic information for gene regions associated with resistance to new and repurposed drugs.

Most importantly, the information provided by tNGS can be crucial to differentiate relapse from new infection, especially in case of strain selection during culture. Therefore, it is necessary to collect samples throughout therapy to enable comparative analysis in case of relapse or reinfection after therapy end. Although WGS and tNGS technologies are mostly limited to high-level, high-throughput testing centers with sufficient infrastructure and well-trained staff to perform these assays, they are both powerful tools for treatment response monitoring and provide key information for any anti-TB treatment response trial. New user-friendly and low-cost platforms such as the Oxford Nanopore Technologies Limited (Oxford, UK) MinION sequencer have the potential to move sequencing closer to point of need, supporting the management of people affected by TB and drug-resistant TB (140). The COVID-19 pandemic has increased the capacity to perform sequencing in a wide variety of settings, which also benefits the TB field. Currently, an array of sequencing solutions suitable for district labs are commercialized and on the pathway to WHO review (32).

MISCELLANEOUS

In addition to the previously referenced technologies, there is a variety of TB diagnostic innovations with TB treatment monitoring potential. For example, certain breath and cough tests operate under the principle that expelled air from TB patients harbors unique pathogen biomarkers or volatile organic compounds that can be used to diagnose infection, thereby providing a quick and potentially easy method for patient screening and detection. Although there are a number of developers investigating the potential of this type of technology (141–147), early clinical studies have shown variable results (sensitivity, 74 to 100%; specificity, 11 to 93%). The treatment monitoring and programmatic potential of this type of technology still remains unclear. Several manufacturers are also developing artificial intelligence (AI) algorithms to interpret and quantify images obtained by point-of-care ultrasound (148), sounds recorded with digital stethoscopes, and cough analyzers, and with the severe acute respiratory syndrome coronavirus 2 (SARS-CoV-2) pandemic these tools have been fast tracked with promising results (149).

CONCLUSIONS AND FUTURE DIRECTIONS

TB biomarkers for therapy monitoring have the potential to serve as future surrogates for anti-TB treatment outcome in clinical trials and in routine clinical settings. However, few markers have been prospectively evaluated for this purpose and studies proving their applicability are desperately needed. In particular, studies with rigorous methodology to specifically evaluate treatment response are necessary, since the vast majority of clinical studies of biomarkers have only provided preliminary data, being underpowered, using only surrogate outcomes for treatment response, or failing to include sufficient time points or comparators. Although many markers appear promising for therapy monitoring or as markers for outcome prediction, most studies are not designed to prove the markers' performance. Clinical evaluation in designated biomarker studies is especially important for markers that indicate cure during treatment, which may allow for personalized therapy durations for TB patients and may even identify those at risk for relapse after therapy end. Biomarkers signaling relapse risk would need to clearly identify relapse patients immediately following completion of standard or experimental therapies. It is important to note that most studies reporting clinical outcome as endpoint do not include relapse but rather indirect surrogates for therapy failure. This is especially important for studies evaluating biomarker guided individualized therapy durations, where studies would need to compare standard versus biomarker-guided therapy durations with relapse as most important endpoint. Regardless, long follow-up periods to exclude relapse will be necessary to underscore the potential of any biomarker to serve as a future outcome-defining surrogate.

Despite the promise of biomarkers for TB treatment and outcome monitoring, research in this area has not yet yielded a single marker that can sufficiently or completely substitute for established culture-based markers at any level of the health care

system (32). It is very likely that no single marker can be used to identify all endpoints of interest (e.g., incipient disease, active disease, or cure versus relapse), and it may therefore be more promising to identify a marker's disease stage-specific potential. For example, assays detecting sputum-based markers such as the MBLA are likely suitable indicators of bactericidal activity (i.e., in EBA trials) but might not indicate risk of relapse at the termination of therapy in phase III trials. In addition, a combined marker approach would likely improve the accuracy of individual markers for certain endpoints. In this context, a combination of host, pathogen, and imaging markers may eventually lead to suitable and rapid individual risk assessments. To accelerate the discovery of promising marker combinations, machine learning and artificial intelligence could be applied to large data sets to identify clinically interpretable marker sets or may even be included in individual assessments in the future.

In conclusion, there are a wide variety of TB treatment monitoring and outcome marker candidates currently under development or already on the market. The combination of these markers may be key to the comprehensive assessment of individual risks for various endpoints, and modern computational approaches are very suitable to accelerate marker identification and interpretation.

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Stephen H. Gillespie, M.D., is Professor of Medicine at the University of St Andrews. He has been involved in various aspects of tuberculosis diagnosis and drug development, including the evaluation of new candidate agents *in vitro* and has led trials exploring moxifloxacin with the TB Alliance in REMoxTB, STAND and SimpliciTB. He is one of the Chief Investigators of the PanACEA consortium. He has developed SLIC (Scattered Light Integrating Collector), performing antibiotic susceptibility testing in $<$30 minutes. This innovation won a Longitude Prize Discovery Award the Scottish Life Science Alliance Innovation of the Year 2017. He has also developed the Molecular Bacterial Load Assay for tuberculosis, nontuberculosis mycobacteria and the organisms of COPD. This is an innovative way of detecting, quantifying and determining the viability of bacteria in a single assay to monitor treatment response. Both innovations are in late-stage clinical development.

Morten Ruhwald, M.D., joined FIND in 2019 as Head of the TB programme. He is a medical doctor with over 15 years of professional experience, including 4 in clinical medicine and 12 in research and development in the area of vaccines and diagnostics for TB. Morten has been project lead on several diagnostic tests for latent *M. tuberculosis* infection, including specific skin tests and new simpler in-vitro diagnostics in the IGRA family. He has worked extensively with international stakeholders in translational medicine and product development for poverty-related diseases. Prior to joining FIND, he was the Chief Medical Officer and Head of Human Immunology at the Center for Vaccine Research at Statens Serum Institute, Denmark, and before that he led the TB immunology group at the Copenhagen University Hospital. Morten obtained his medical degree and Ph.D. from Copenhagen University, Denmark. and has published more than 80 academic papers.