Journal of Infection 90 (2025) 106399

Contents lists available at ScienceDirect

Journal of Infection

journal homepage: www.elsevier.com/locate/jinf

Infectious Disease Practice

The molecular bacterial load assay predicts treatment responses in patients with pre-XDR/XDR-tuberculosis more accurately than GeneXpert Ultra MTB/Rif

Marit Neumann [a,b], Maja Reimann [b,c,d], Dumitru Chesov [b,c,e], Cristina Popa [f], Antonela Dragomir [f], Oana Popescu [f], Roxana Munteanu [f], Alexandra Hölscher [a], Isobella Honeyborne [g], Jan Heyckendorf [c,h,i], Christoph Lange [b,c,d,j], Christoph Hölscher [a,b,*,1], Barbara Kalsdorf [b,c,1]

[a] Division of Infection Immunology, Research Center Borstel, Parkallee 1–40, 23845 Borstel, Germany
[b] German Center for Infection Research (DZIF), Partner site Hamburg-Lübeck-Borstel-Riems, Borstel, Germany
[c] Division of Clinical Infectious Diseases, Research Center Borstel, Parkallee 1–40, 23845 Borstel, Germany
[d] Respiratory Medicine & International Health, University of Lübeck, Lübeck, Germany
[e] Nicolae Testemitanu State University of Medicine and Pharmacy, Stefan cel Mare si Sfant Boulevard 165, MD-2004 Chisinau, Republic of Moldova
[f] National Institute of Pneumology Marius Nasta, Şoseaua Viilor 90, 050159 Bucharest, Romania
[g] Centre for Clinical Microbiology, University College London, Gower Street, London WC1E 6BT, United Kingdom
[h] Internal Medicine I, University Hospitals Schleswig-Holstein, Kiel, Germany
[i] Pulmonology and Inflammatory Medicine, Christian Albrechts-University, Kiel, Germany
[j] Baylor College of Medicine and Texas Children´s Hospital, Houston, TX, USA

ARTICLE INFO

Article history:
Accepted 22 December 2024
Available online 27 December 2024

Keywords:
Drug-resistant tuberculosis
Pulmonary tuberculosis
Diagnostic evaluation
Molecular bacterial load assay (MBLA)

SUMMARY

Objectives: Early detection of treatment failure is essential to improve the management of drug-resistant tuberculosis (DR-TB). We evaluated the molecular bacterial load assay (MBLA) in comparison to standard diagnostic tests for monitoring therapy of patients affected by drug-resistant TB.
Methods: The performance of MBLA in tracking treatment response in a prospective cohort of patients with pulmonary MDR/RR- and pre-XDR/XDR-TB was compared with mycobacterial culture, mycobacterial DNA detection using GeneXpert (Xpert) and microscopy detection of sputum acid-fast-bacilli.
Results: *Mycobacterium tuberculosis* culture conversion was used as the read-out for treatment responses. The MBLA was most concordant during the early phase of treatment, detecting changes in bacterial load with similar accuracy to microscopy and outperforming Xpert. When considering all timepoints, concordance with MGIT results was 72.1% for MBLA, 57.4% for Xpert and 76.7% for microscopy. The AUC for culture conversion was higher for MBLA (0.88, CI 0.84–0.95) than for Xpert (0.78, CI 0.72–0.85) and microscopy (0.77, CI 0.71–0.83).
Conclusions: MBLA was superior in the early identification of successful culture conversion compared to microscopy and Xpert and could be a useful biomarker to evaluate novel entities in Phase IIA early-bactericidal-activity drug trials regardless of the degree of *M. tuberculosis* drug resistance.

Introduction

Tuberculosis (TB) is a leading cause of morbidity and mortality world-wide. The World Health Organization (WHO) estimates that there were 10.6 million incident TB cases in 2022 and approximately, 1.3 million people died from the disease.[1] The standard treatment for drug-susceptible pulmonary TB consists of initial therapy with isoniazid, rifampicin, pyrazinamide and ethambutol for a period of two months, followed by isoniazid and rifampicin for an additional four months.[2] Drug-resistant (DR) strains of *Mycobacterium tuberculosis* have emerged over the past decades and are now threatening TB control in many high-burden countries.[3,4] Drug resistant *M. tuberculosis* is classified into rifampicin-mono-resistant TB

* Correspondence to: Research Center Borstel, Parkallee 1–40, 23845 Borstel, Germany.
E-mail address: choelscher@fz-borstel.de (C. Hölscher).
[1] Shared last authorship.

https://doi.org/10.1016/j.jinf.2024.106399

(RR-TB), multidrug-resistant TB (MDR-TB; defined by bacillary resistance against rifampicin and isoniazid). In 2020 further classifications were introduced, namely, pre-extensively drug-resistant TB (pre-XDR-TB; MDR/RR-TB plus resistance against any fluoroquinolone) and extensively drug-resistant TB (XDR-TB; defined by pre-XDR-TB plus additional resistance to the other WHO group A medicines bedaquiline and/or linezolid).[5–7] Treatment outcome for patients affected by DR-TB has been less favorable compared to treatment of drug-susceptible TB at programmatic levels.[1,8,9] To better control DR-TB, early detection of treatment failure is essential. Therefore, diagnostic tools that are suited to monitor treatment responses that can inform therapy response in real-time are urgently needed.

Pulmonary TB is confirmed by growth of *M. tuberculosis* in liquid or on solid media from bronchopulmonary specimens.[10,11] To monitor treatment response during anti-TB therapy, sputum is analyzed longitudinally for the presence of *M. tuberculosis*. The decline of acid-fast bacilli (AFB) measured by sputum microscopy gives an indication of treatment response. However, microscopy cannot distinguish between viable and dead mycobacteria or differentiate *M. tuberculosis* and non-tuberculous mycobacteria. Most importantly the sensitivity is poor (samples containing $< 10^4$ bacilli ml^{-1} are likely to be undetected) and dead bacteria are still visible in microscopy even after successful culture conversion.[12,13] Therefore, culture conversion from viable bacilli to a negative culture for two consecutive timepoints is used as the gold-standard to indicate treatment success. The main disadvantage of culture conversion, however, is the time delay, as cultures must remain negative in liquid or on solid media for 42 or 56 days, respectively. For this reason, a decision to change the therapeutic regimen may only be made at a relatively late stage in the event of treatment failure. Cultures also become contaminated with other microorganisms leading to invalidation and therefore loss of data[14] and they are unable to detect live but non-culturable bacteria.[15] This delay is particularly critical for individuals with DR-TB where the full pattern of drug resistance may not be known.

Detection of *M. tuberculosis*-specific nucleic acid reduces the time-to-result from days to hours. In addition to microbiological methods of microscopy and culture, nucleic acid amplification techniques are applied to the detection of *M. tuberculosis* from direct biospecimens or cultures.[16] The most widely used method is the GeneXpert / GeneXpert Ultra (Xpert; Cepheid, CA)) technology which allows the detection of *M. tuberculosis* genomic DNA within 90 min. The WHO recommends Xpert for rapid detection of *M. tuberculosis* from biospecimens as an initial diagnostic test in individuals suspected of being infected with *M. tuberculosis*. Although identification of *M. tuberculosis* genomic DNA by Xpert is highly sensitive, it has been found to be a poor measure of mycobacteria viability.[17–21] For this reason, Xpert is not recommended to monitor treatment responses in TB.[17,20]

The molecular bacterial load assay (MBLA) is a culture-free test used to quantify *M. tuberculosis* 16S rRNA as a surrogate marker of viable bacilli using the reverse transcriptase polymerase chain reaction (RT-PCR).[17] Changes in bacterial load, measured using the MBLA have been demonstrated to correlate closely with colony counts on solid culture during early response of DS-TB to treatment[22] and it is suggested that the MBLA can detect both, replicating and dormant but viable *M. tuberculosis*.[19] Therefore, the MBLA has potential to detect the treatment response of DR-TB early during treatment. However, while a comprehensive comparison with microscopy, Xpert and culture has shown that MBLA is able to ascertain treatment responses in drug-susceptible TB,[23] a systematic evaluation of MBLA to monitor treatment responses in patients affected by DR-TB has not been performed to date.

Here we evaluated the performance of the MBLA as a treatment monitoring biomarker in a prospective cohort of adult patients with pulmonary MDR/RR- and pre-XDR/XDR-TB in comparison to detection of AFB by microscopy, mycobacterial culture and the detection of *M. tuberculosis*-specific DNA by Xpert from sputum specimens.

Methods

Study participants

From 1st August 2018 to 30th April 2019, we prospectively enrolled adult patients with genotypically confirmed RR pulmonary TB (detected by Xpert on sputum specimens). These individuals were treated on the DR-TB ward at Marius Nasta Pneumology Institute in Bucharest, Romania, a tertiary level medical institution specializing in medical care of patients with respiratory diseases. Participants were either treatment naïve or had received less than 5 days of anti-TB therapy during the current TB episode.

Bacillary resistance was subsequently determined by culture and assigned the resistance pattern of MDR/RR-TB, pre- XDR-TB/XDR-TB (Fig S2) as defined by the current WHO definition.[7]

Serial sputum samples were collected at enrollment and bi-weekly for the duration of treatment (unless participants were unable to produce sputum). At each time point, two independent sputum samples were collected. One sample was immediately tested by sputum smear microscopy for detectable AFB; Xpert for detectable *M. tuberculosis* DNA and culture growth of *M. tuberculosis* in Mycobacterial Growth Indicator Tubes (BD BACTEC MGIT 320, Becton, Dickinson and Company, East Rutherford, USA). The second sample was stored at −80 °C for later analysis with MBLA. Complementary to these tests, basic clinical and demographic data of the participants were recorded. All data were anonymized, and reporting followed the STARD criteria.[24]

After detection of *M. tuberculosis* DNA and genotypic prediction of rifampicin resistance by Xpert, treatment for drug-resistant TB was initiated with a later generation fluoroquinolone, a second line injectable drug (amikacin, capreomycin or kanamycin) ethionamide, cycloserine or para-aminosalicylic acid. As soon as phenotypic drug susceptibility test (DST) results became available, the treatment regimen was adapted accordingly. The choices of treatment regimens followed the recommendations of the Romanian National TB Guidelines.[25]

Microscopy

Sputum was stained with auramine–rhodamine to visualize AFB by fluorescence microscopy at Marius Nasta Pneumology Institute at Bucharest. Based on WHO criteria results were classified into five categories: negative, scanty, 1+, 2+ and 3+.[26]

M. tuberculosis -culture and DST

Prior to bacterial culture, sputa were decontaminated at the Marius Nasta Pneumology Institute in Bucharest, Romania, using 4% sodium hydroxide and 0.067 mol/l phosphate buffer at pH 6.8, following the modified Petroff method.[27] Cultivation was performed using Mycobacteria Growth Indicator Tubes (MGIT, BD) to detect growth of *M. tuberculosis*. The time to culture positivity (TTP) was determined in days. Phenotypic drug susceptibility testing (DST) of *M. tuberculosis* was performed at the national reference laboratory for mycobacteria at the Marius Nasta Pneumology Institute in Bucharest, Romania. The drugs included were rifampicin, isoniazid, ethambutol, streptomycin, ofloxacin, amikacin, kanamycin and capreomycin.

Mycobacterial load assay (MBLA)

Sputum of varying quantities (2 −14 ml) to be used for RNA extraction was stored in tubes at −80 °C at Marius Nasta Pneumology Institute without any further processing. In the biosafety level 3 (BSL3) laboratory, Research Center Borstel, Germany, samples were thawed in batches at room temperature for RNA extraction. For each

batch, 2 control samples were processed together with the sputum samples, one of which contained *M. bovis* Bacille Calmette-Guérin (BCG) as a positive control. To aid further processing of the sputum samples mucolysis sputum digestant (Pro-Lab diagnostics, Bromborough, UK) was used and prepared according to the manufacture's instructions. To each sputum sample the same amount of mucolysis sputum digestant was added and vortexed for 30 s. After 15 min of incubation at room temperature, samples were centrifuged at 2000xg for 10 min. If the sputum and mucolysis reagent were not homogeneous, the procedure was repeated. Five ml guanidine thiocyanate buffer was added per 1 ml of the remaining sample and an internal control was added as published.[17] Afterwards, RNA was extracted as previously described.[17] Expression of 16S rRNA was measured using reverse transcriptase quantitative PCR performed on a LightCycler 480 II (Roche, Basel, Switzerland) as described.[17] Primers and probes used were purchased from Eurofins Genomics (Ebersberg, Germany). All other reagents were from the Quantitect Multiplex RC-PCR kit (Qiagen, Hilden, Germany). *M. tuberculosis* quantification cycle value (Cq was normalized based on the equation *M. tuberculosis* Cq - [(control Cq −20.00) x 0. 9239] which was calculated based on our data (Fig. S1). We converted the bacterial load in sputum from the normalized Cq-value using a standard curve which we previously generated using different concentrations of culture-derived bacteria. The limit of detection (LOD) was determined based on spiked numbers of *M. bovis* BCG into sputum and those samples with less than 5×10^2 bacteria per ml were considered negative. The readout was adjusted to reflect the initial sputum volume. Based on the relationship between bacterial load and the normalized Cq value, we determined the normalized Cq value per ml and used this to determine the bacterial load per ml.

Xpert

Xpert was performed according to the manufacturer's instructions (Cepheid, Cepheid, Sunnyvale CA, USA)[28,29] at Marius Nasta Pneumology Institute at Bucharest, Romania.

Statistical analysis

Categorical variables were reported using counts and percentages and continuous variables using medians and interquartile ranges (IQR). For the test performance analysis, the reference standard was considered *M. tuberculosis* -culture positivity. To facilitate statistical analysis and the comparison of MBLA with MGIT and Xpert we used normalized Cq per ml instead of the bacterial load per ml for MBLA. For comparative analysis between groups, the Mann-Whitney U-test for nonparametric data was used. Statistical tests for paired data were performed by Wilcoxon Signed Rank test. A p-value of < 0.05 was considered significant. Spearman's rank correlation was used to evaluate the linear relationship between the MBLA and the Xpert compared to TTP in MGIT.

We considered bacterial loads determined using the MBLA above the median at baseline as 'high bacterial load' and 'low bacterial load' for those below the median. AFBs of grade 2+ and 3+ at baseline were categorized as 'high AFB' while those with grade 1+, scanty or negative were regarded as 'low AFB'. Median percentages of affected lung tissue in X-ray were calculated and hereby patients were classified in two groups with a 'large proportion' or a 'small proportion' of lung tissue affected. Differences in time to culture conversion by participants with high-baseline versus low-baseline in bacillary load or detectable AFBs on microscopy, different HIV status, cavities or no cavities by X-ray, affected lung tissue, sex and resistance profiles were evaluated using the log-rank test. Probability values < 0.05 were considered statistically significant. Statistical analysis was performed using Graphpad Prism version 9 (Graphpad Software Inc., San Diego, California, USA) and R version 4.3.1.[30] Concordance correlation coefficient was calculated using epiR package in R. For determination of ideal cutoff values for the prediction off culture conversion we used the cutpointr package in R.

Ethics

The study protocol was positively evaluated by the Ethics committee of the University of Lübeck (Lübeck, Germany; AZ 18–217) and Marius Nasta Pneumology Institute in Bucharest, Romania (5534).

Results

Demographics and characteristics of study subjects

In total, 36 individuals were screened for enrollment into the study. Of those, 3 were excluded when *M. tuberculosis* did not grow in their sputum cultures. Another 4 did not have a culture result available to confirm DR-TB and were also excluded. In total, 29 individuals met the inclusion criteria and accepted to be enrolled (Fig. 1). The median age of the patients was 42 (IQR 31.5–57.5) years. The minority was female (24.1%). Fifteen of 29 (51.8%) had a previous history of TB and 21/29 (72.4%) were active smokers. Five of 29 (17.2%) were people living with HIV (PLHIV); 21/29 (72.4%) were HIV-seronegative and 3/29 (10.3%) had an unknown HIV-status. Two of 29 (6.9%) had Hepatitis C-infection, 2/29 (6.9%) were on chemotherapy for a malignancy and 1/29 (3.4%) was diabetic. On chest radiographs 25/29 (86.2%) had cavitary lesions. The median percentage of lung lesion extent by radiography was 50% (30–70%). Baseline characteristics of the participants are given in Table 1.

Three of 29 (10.3%) participants were infected with a RR strain of *M. tuberculosis* without isoniazid resistance, 14/29 (48.3%) had MDR-TB and 10/29 (34.5%) had pre-XDR/XDR-TB (due to the unavailable bedaquilin- and linezolid-resistance status it was not possible to differentiate between pre-XDR and XDR). In 2/29 (6.9%) DST-data were only available for rifampicin and isoniazid. Resistance pattern and treatment regimens are shown in Fig. S2. At diagnosis, all participants (29/29) had culture confirmed DR-TB and genotypic proof of *M. tuberculosis* measured with the MBLA and Xpert (Fig. 1). AFB were microscopically detectable in 23/29 (79.3%) sputum samples (Table 2), 6 with AFB grade scanty, 8 with AFB grade 1 (+), 7 with AFB grade 2 (++), and 2 with AFB grade 3 (+++) (detailed information about smear microscopy results can be found in Fig. S3).

Two participants (2/29; 6.9%) died of TB in the first 42 days of treatment and were therefore excluded from the follow-up analysis and stratification for culture conversion. The median time of follow-up in the 27 participants was 14 weeks (IQR 10 - 16) with a minimum of 4 and a maximum of 24 weeks.

MBLA, Xpert and microscopy at culture conversion

Twenty-four of 27 (88.9%) reached culture conversion at a median time of 42 days of treatment (IQR 20–80.5) and were classified among the group of participants with successful culture conversion. In comparison to MGIT as gold standard at the last time point 7/24 (29.2%) were also diagnosed with a negative MBLA, a negative Xpert as well as a negative microscopy result. Nine of the 24 (37.5%) had a negative MBLA result but were still positive by Xpert. Out of these 9 participants, 8 had undetectable AFBs on sputum smear microscopy but 1 had a positive smear result for the last available culture. One of 24 (4.2%) was positive by MBLA but negative by Xpert and microscopy. Seven of 24 (29.2%) with culture conversion were still positive by MBLA and Xpert. Of those, one had a positive result for microscopy, the other six were considered smear negative. Overall MBLA classified 16/24 (66.7%) with culture conversion in MGIT as true negative while Xpert classified 8/24 (33.3%) as true negative. Microscopy identified 22/24 (91.7%) as negative, of

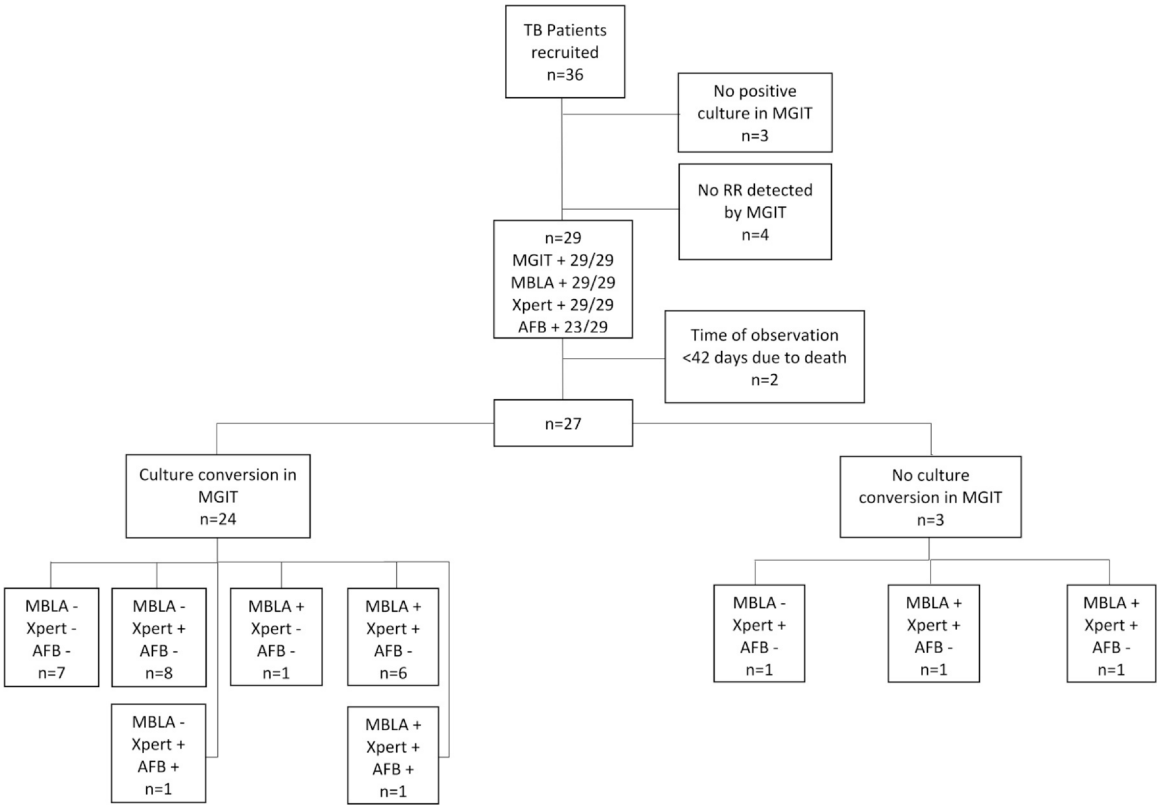

Fig. 1. Flow chart of patients included in the study. TB= tuberculosis; MGIT =Mycobacteria growth indicator tube; MBLA= Molecular bacterial load assay; RR = Rifampicin resistance.

Table 1
Demographics and characteristics of study subjects.

Characteristics	N (%) or Median (IQR)
Age at enrollment	42 (31.5–57.5)
Female Gender	7 (24.1)
Romania as Country of birth	29 (100)
Current smoker	21 (72.4)
Drinking alcohol more than monthly	16 (55.2)
Immunosuppression	
Diabetes	1 (3.4)
Chemotherapy	2 (6.9)
HIV	
Positive	5 (17.2)
Unknown status	3 (10.3)
Hepatitis B	0(0)
Hepatitis C	2 (6.9)
Silicosis	0(0)
Organ transplant	0(0)
Previous history of TB	15 (51.7)
Karnofsky Score	90 (80–90)
X-Ray characteristics	
Cavities in X-Ray	25 (86.2)
Affected lung percentage	50 (30–70)
TTP at diagnosis (days)	17 (13–22)
M. tuberculosis - Resistance	
Rifampicin resistant TB	3 (10.3)
MDR-TB	14 (48.3)
Pre-XDR/XDR-TB	10 (34.5)
Undetermined	2 (6.9)
Time point of culture conversion (days)	42 (28–80.5)
Last sputum available (days)	98 (63–119)

IQR= interquartile range; HIV= human immunodeficiency virus; TB= tuberculosis; TTP= time to culture positivity; MDR-TB= multidrug-resistant TB; XDR-TB= extensively drug-resistant TB.

which 6/24 (25%) already had a negative test result at baseline. Three out of 27 (11.1%) did not reach culture conversion after 56, 70 or 112 days of treatment. Of those participants with continuous detectable *M. tuberculosis* growth, 1/3 (33.3%) was positive only by Xpert, 1/3 (33.3%) had a positive test result with the MBLA and Xpert but not by microscopy and 1/3 (33.3%) was considered positive by all three tests. In summary, and in comparison to the gold standard MGIT, MBLA identified 18/27 (66.7%) and Xpert 11/27 (40.7%) results while microscopy was concordant on 23/27 (85.2%) occasions at the last available culture.

Change in bacterial load measured by MGIT, MBLA, Xpert and microscopy during treatment follow -up

At diagnosis the median time to positivity (TTP) was 12 (IQR 8–15) days (Fig. 2A). After 2 weeks of treatment the TTP increased significantly to 18 days (p= < 0.0001), this further increased to 22 days after 4 weeks of treatment and culture conversion was reached after a median of 42 (28–80.5) days of treatment (Table 1). At the time of diagnosis, the median Cq value measured by the MBLA was 18.66 cycles and corresponded with a bacterial load of 1.87×10^6 CFU/ml sputum. Cq-results increased significantly during the first two weeks of treatment (23.22 cycles, equal to a bacterial load of 9.05×10^4 CFU/ml sputum, p= < 0.0001) and between the second to the fourth week of treatment (27.09 cycles, corresponding with a bacterial load of 6.92×10^3 CFU/ml sputum, p = 0.0001), the rate of change in Cq slowed down (Fig. 2B). Xpert responded more slowly and increased from a median Cq of 16.3 to 16.5 to 17.4 in the first 4 weeks (Fig. 2C), without any continuous trend for the following weeks. Microscopy revealed a rapid response to therapy (Fig. 2D), with most samples showing no evidence of AFB after 4 weeks.

All tests displayed a response to treatment resulting in a decrease of test positivity rate (Fig. 2E). The MBLA showed a slightly slower decrease than MGIT culture, while decrease in smear positivity measured by microscopy was fastest in the first weeks and then slowed down. For Xpert the decrease was noticeably slower than in

Table 2
Microscopy results.

AFB N (%)	negative	scanty	+	++	+++
at diagnosis (n=29)	6 (20.7)	6 (20.7)	8 (27.6)	7 (24.1)	2 (6.9)
at culture conversion (n=27)	19 (79.2)	4 (16.7)	1 (4.2)	0	0
last sputum available (n=27)					
with culture conversion (n=24)	22 (91.7)	1 (4.2)	0	1 (4.2)	0
without conversion (n=3)	2 (66.7)	1 (33.3)	0	0	0

AFB= acid fast bacilli; Two participants (2/29; 6.9%) died of TB in the first 42 days of treatment and were therefore excluded from follow-up analysis and stratification for culture conversion.

the other tests with most samples still being positive after 14 weeks of treatment.

MGIT, MBLA, Xpert and microscopy -results stratified by culture conversion

Stratifying test results by culture conversion in MGIT with corresponding MBLA, Xpert and microscopy results led to four categories: the green symbols in Fig. 3 depict all participants, who ended treatment with culture conversion and their corresponding results in MBLA, Xpert and microscopy. Cq measured by the MBLA increased similarly to TTP. Cq of Xpert lagged behind, whereas microscopy grading showed the same rapid response to therapy as MGIT and MBLA. In the 3 participants, who did not achieve culture conversion during follow-up (red symbols in Fig. 3), the TTP increased but did not reach culture conversion. The Cq measured using the MBLA as well as microscopy followed the same trend, whereas the Cq of Xpert remained positive. In 8/24 participants with culture conversion, the MBLA still detected viable bacteria (in comparison to MGIT; here defined as false positive MBLA; blue symbols in Fig. 3). MBLA-measured Cq had a corresponding increase when compared to MGIT and microscopy, but Cq-values stayed slightly beneath the limit of detection. Xpert Cq-values remained around 20 cycles for the complete treatment course. At the time point of the last available culture, 16/24 patients with culture conversion had a positive Xpert result (here defined as false positive Xpert; brown symbols in Fig. 3): Cq measured using the MBLA showed a similar course as TTP, increasing Cq-values during treatment and in nine of those 16 patients Cq crossed the limit of detection resulting in a negative MBLA result (see also Fig. 1). Microscopy results were similar, with more patients converting (see also Fig. 1). The other 7 patients had a Cq close to the detection limit (median Cq 27.73, IQR 26.85 to 29.88). Whereas Xpert stayed positive with a high Cq-value ~ 20 for the whole treatment course.

MBLA and Xpert results stratified by MGIT positivity/negativity as violin plots (Fig. S4) depict that in culture-negative TB MBLA correlated with a high Cq-value, whereas Xpert still detected higher bacterial burden (low Cq-values).

Overall, the stratification of the results revealed a comparable identification of converters and non-converters when measured by MGIT, the MBLA and microscopy.

Clinical utility of MBLA in comparison to Xpert and microscopy for probability of culture conversion

To determine correlation between the MBLA and MGIT, respective Xpert and MGIT as well as microscopy and MGIT, normalized Cq/ml of the MBLA (Fig. 4A) or Cq-values from the Xpert test (Fig. 4B) as well as microscopy results (Fig. 4C) for all time points were used as a continuous variable to correlate with TTP of MGIT. Spearman rank had correlation values of 0.73, 0.54 and –0.61 respectively. Cohens Kappa was 0.42 (95% CI: 0.3 - 0.55) for the MBLA, 0.14 (95% CI: 0.04 - 0.24) for Xpert and 0.54 (95% CI 0.42 - 0.66) for microscopy.

In the concordance analysis of binary results (testing positivity or negativity, Fig. 5) both the MBLA (Fig. 5A) and Xpert (Fig. 5B) had very high concordance with MGIT culture at baseline while concordance was lower for detectable AFB on microscopy (Fig. 5C). Later the concordance of Xpert and MGIT decreased noticeably resulting in an overall concordance of 57.4%. The concordance of the MBLA and microscopy with MGIT culture each remained constant over the course of treatment. Overall concordance was slightly higher in microscopy (76.7%) than in MBLA (72.1%). The analysis of the concordance of the individual test methods with the gold standard MGIT therefore revealed that both, MBLA and microscopy had, over the course of treatment, a similar degree of positive and negative findings confirmed in culture.

The comparison of the receiver operating characteristics (ROC) curve analysis for the MBLA, Xpert and microscopy in comparison to culture as the gold standard revealed that the MBLA had higher ability to act as a surrogate biomarker for culture conversion (Fig. 6). The AUC was higher for the MBLA (AUC 0.88, 95% CI 0.84 – 0.95) than for Xpert (AUC 0.78, 95% CI 0.72 – 0.85) and microscopy (AUC 0.77

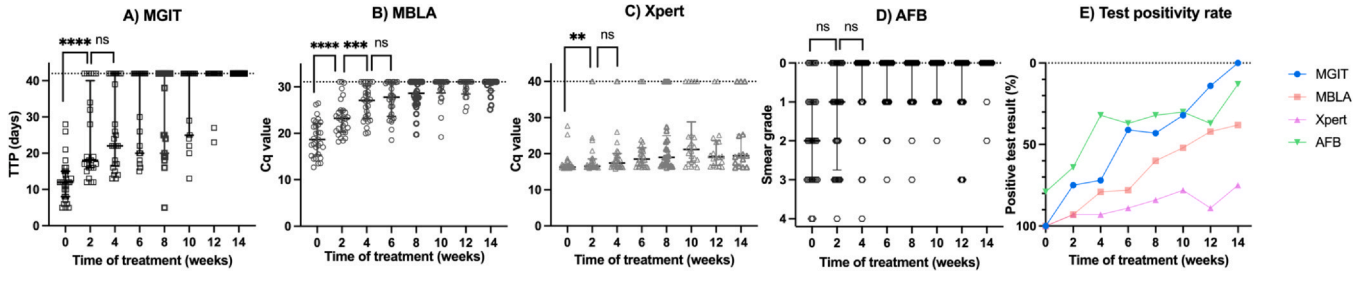

Fig. 2. Changes in MGIT, MBLA, Xpert and microscopy during treatment. Biweekly change of bacterial load measured as time to culture positivity (TTP) in days in mycobacterial growth indicator tube (MGIT) (A), change of Ct-values in molecular bacterial load assay (MBLA) (B), GeneXpert MTB/RIF Ultra (Xpert) (C) and microscopy (D) in 29 patients. Values are depicted until the median time point of last available culture result in this study. Bars represent median and interquartile range. Statistical comparisons using Wilcoxon signed Rank test were made for before treatment and week 2 as well as between week 2 and 4. For MBLA additional comparison between the respective subsequent measurement points was performed until there was a non-significant change. P-values < 0.05 were considered statistically significant. Tests' positivity rates over the course of treatment until median time of last available culture (E) are shown for culture results by MGIT, detection of viable bacteria by MBLA, detection of M. tuberculosis DNA by Xpert and detection of acid-fast bacilli (AFB) from sputum specimen.

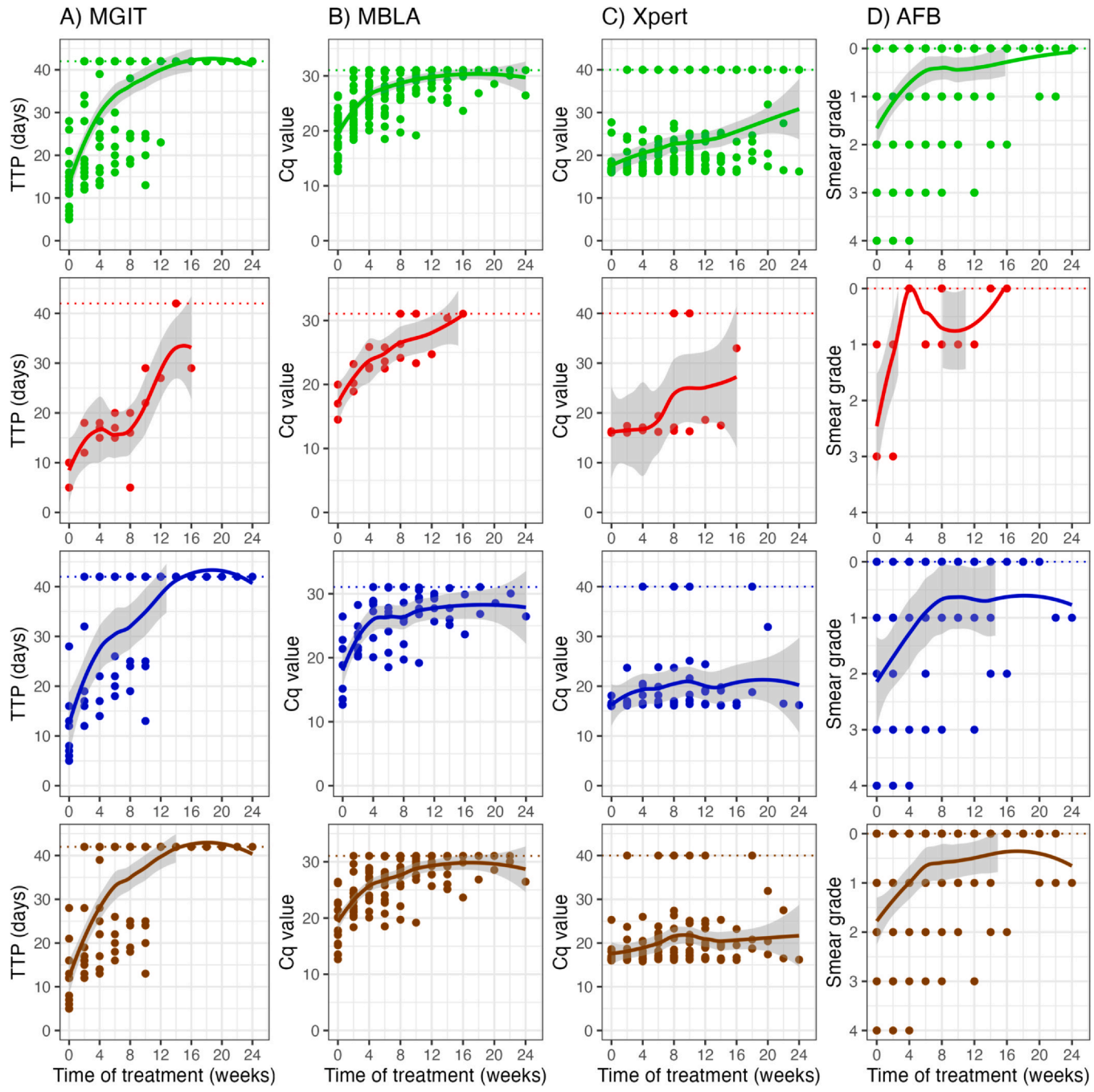

Fig. 3. MGIT, MBLA, Xpert, and microcopy -results stratified by culture conversion. Depending on culture conversion in MGIT patients were classified as successfully culture converted (n=24, green) or not successfully culture converted (n=3, red). The rows below depict values for patients who reached culture conversion in MGIT but were classified as positive by either MBLA (n=8, blue) and Xpert (n=16, brown) at the point of last available culture result. Graphs show the change in bacterial load over the course of treatment in these four groups by (A) Mycobacteria growth indicator tube (MGIT), (B) Molecular bacterial load assay (MBLA), (C) GeneXpert MTB RIF/ Ultra (Xpert), and (D) microscopy (AFB). The line depicts mean values with the surrounding grey area showing standard deviation with single values appearing as points. The dotted line reflects the limit of detection.

95% CI 0.71 - 0.83). Combined AUC for Xpert and microscopy was 0.82 (95% CI 0.75 - 0.88) and combined AUC of MBLA and microscopy 0.9 (95% CI 0.85 - 0.94). At the optimum cut-off for MBLA with a Cq-value of 26.45 patients close to culture conversion were identified with a sensitivity and a specificity of 93.9% and 76.0% respectively. At the optimum Cq value for Xpert with Cq=18.2 the sensitivity was 77.2% and the specificity 78% while sensitivity and specificity for microscopy were 66% and 85.6% respectively. Combined test parameters were higher in Xpert + microscopy (sensitivity 85.6%, specificity 65%) and MBLA + microscopy (sensitivity 87.3%, specificity 80%). From this analysis it can be concluded that both MBLA alone and MBLA combined with microscopy were able to perform optimally in terms of sensitivity and specificity to detect viable bacilli without including a culture readout.

Probability of culture conversion in regard to resistance level

The cohort was stratified for severity of phenotypical resistance pattern and analyzed for non-conversion over the time course of treatment in patients who provided sufficient DST- data (Fig. 7). The median duration of treatment until culture conversion for participants with pre-XDR/XDR-TB was 84 days (IQR 70 to 84 days) in

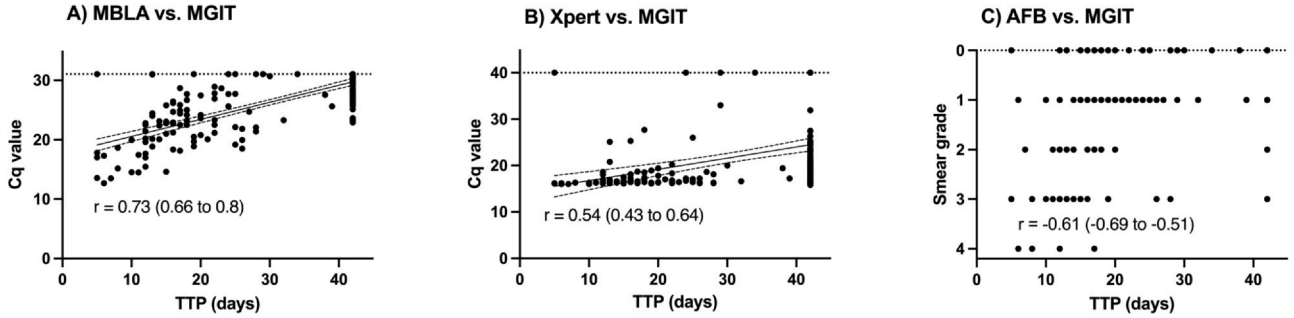

Fig. 4. Correlation of MGIT with MBLA, Xpert, and microscopy over all time points. Plots of (A) MGIT (TTP in days) against MBLA (Cq) (n=197), (B) MGIT (TTP in days) against GeneXpert (Cq) (n=197), and (C) MGIT (TTP in days) against microscopy (n=197) in all samples. For analysis all samples provided by individuals included in the study that provided a valuable result for MGIT and at least one other Xpert, MBLA or microscopy were used. Spearman's correlation coefficients (r) with 95% confident interval are presented in the figure. Best-fit linear regression lines are plotted with 95% confidence intervals. The dotted line depicts the limit of detection. MGIT= Mycobacteria growth indicator tube; MBLA= Molecular bacterial load assay; Xpert = GeneXpert MTB/ RIF Ultra; AFB = acid-fast bacteria identified by microscopy; TTP = Time to positivity; Cq = Quantification cycle value.

comparison to a significantly shorter median time in patients with MDR/RR-TB (median 42 days, IQR 14 to 56) (Fig. 7A). This effect was similarly seen in the time to sputum conversion in the MBLA (Fig. 7B) and Xpert (Fig. 7C) but not in microscopy (Fig. 7D). The effect of treatment appears much later in Xpert in comparison to the decline of MGIT TTP, the MBLA and microscopy. The numbers of those who did not successfully convert were too small to investigate, as to whether the effect of early treatment response could be detected using the MBLA. The comparison of the median duration of treatment until culture conversion measured by the individual tests and related to the type of antibiotic resistance showed that the MBLA determines treatment success very early during therapy in both forms of drug-resistant TB.

Discussion

This study evaluates the performance of the MBLA, Cq by Xpert and AFB by microscopy in determining the bacterial load of mycobacteria for the diagnosis of TB and for monitoring of treatment responses in sputum samples from patients with pulmonary DR-TB in comparison with TTP in liquid culture by MGIT. In therapy-naïve patients, the MBLA was more sensitive than microscopy for the diagnosis of TB. Later during therapy it detected changes in bacterial load with similar accuracy to microscopy, and notably more accurately than Xpert, thereby reducing the time to results for treatment response from weeks by culture to hours. MBLA was most concordant with culture results in the early phase of treatment and was indicative of the level of drug resistance.

When treating individuals with DR-TB, early detection of failing therapy is of great importance. However, current standard methods for determining treatment success are either slow or imprecise. The MBLA is a novel molecular method to identify viable *M. tuberculosis* in biological specimens with the advantages of speed, accuracy and a low sample failure rate.[17,31,32] The MBLA improves current standard microbiological assessments for monitoring the treatment of TB. To date, few studies have investigated MBLA performance in comparison to other biomarkers such as *M. tuberculosis* culture conversion or the detection of *M. tuberculosis*-specific DNA by Xpert and evaluations are almost exclusively restricted to patients with drug-susceptible disease.[22,33] Until now the MBLA had only been evaluated to determine the early bactericidal activity (EBA) in one DR-TB drug-trial.[19] In the present study, we therefore compared the performance of the MBLA as a biomarker to ascertain anti-TB treatment success in comparison to culture conversion in sequential sputum samples from patients with pulmonary DR-TB under therapy.

The MBLA predicted *M. tuberculosis* culture-conversion in MGIT with high accuracy. The assay detected treatment response after 2 to 4 weeks of therapy and displayed an early significant decrease in bacterial load. The finding of this early decline of mycobacterial number, measured by the MBLA correlated with TTP as determined in MGIT and was consistent with studies that included participants with drug-susceptible TB in which MBLA was introduced as a marker for EBA to monitor early treatment response.[22,34] The present study

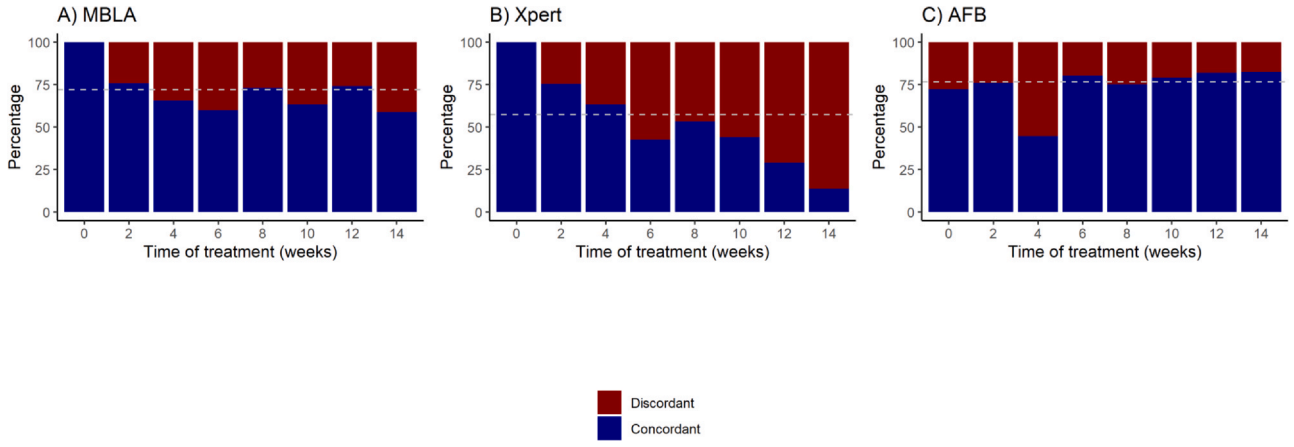

Fig. 5. Test concordance results regarding MGIT culture positivity and negativity. Graphs show the proportion of concordance (blue) and discordance (red) regarding binary results (test positive or negative) for (A) MBLA (n= 197), (B) Xpert (n=197) and (C) microscopy (n=197) for each time point during the median time of observation when compared to MGIT test results. Overall concordance is depicted as the dotted line. MGIT =Mycobacteria growth indicator tube; MBLA= Molecular bacterial load assay; Xpert = GeneXpert MTB/RIF Ultra; AFB= acid fast bacilli.

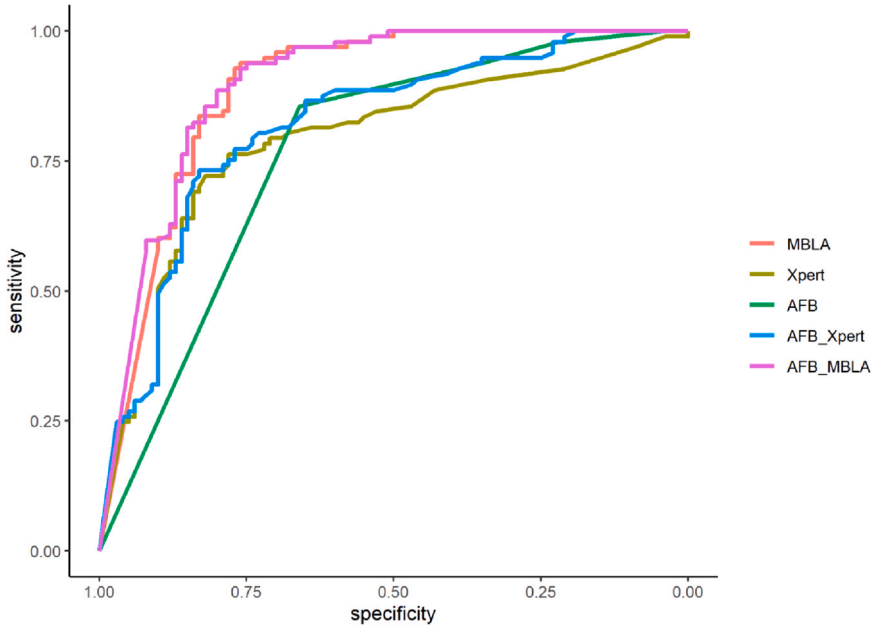

Fig. 6. ROC-curve analysis of MBLA, Xpert, microscopy, MBLA +microscopy and Xpert +microscopy in regard to culture conversion. For analysis all samples that provided a valuable MGIT result and results for MBLA, Xpert and microscopy (n=197) were included. MBLA= Molecular bacterial load assay; Xpert = GeneXpert MTB/RIF Ultra; AFB= acid fast bacilli; ROC = Receiver Operating characteristics; AUC= Area under the curve.

extends the measurement beyond EBA to provide a comparison of the performance of the MBLA to other methods for *M. tuberculosis* detection until the point of culture conversion. A normalized MBLA-Cq-value of 26 corresponded to culture conversion with 94% sensitivity and 76% specificity strongly suggesting that the MBLA can serve as a real-time biomarker for culture conversion thus indicating treatment success. Though limited in number of cases, our study shows that no decline of MBLA-Cq-values during therapy is highly indicative of treatment failure and much earlier compared to *M. tuberculosis* culture results. It should be noted that culture, although considered the gold standard, will in some cases fail to grow viable bacilli. This is due to several reasons, including sodium hydroxide decontamination of sputum, killing some bacteria and dormant bacteria that have been shown to require resuscitation promotion factors for growth.[35,36] Therefore, the specificity of the MBLA may be underestimated in the present study. Despite these limitations our data confirm the utility of the MBLA to indicate an unfavorable outcome at an early stage of treatment.[32]

In contrast to the MBLA, Xpert was less concordant in reference to MGIT as gold standard confirming the slow response by Xpert in comparison to the MBLA, as observed in another study.[34] The reason is believed to be that MBLA detects rRNA exclusively from viable *M. tuberculosis*, while Xpert detects DNA from both viable and dead *M. tuberculosis* cells.[17–21] In support of this, the kinetics of our MBLA test results more closely resembled the time course of *M. tuberculosis* detection in culture during treatment than the kinetics of Xpert.

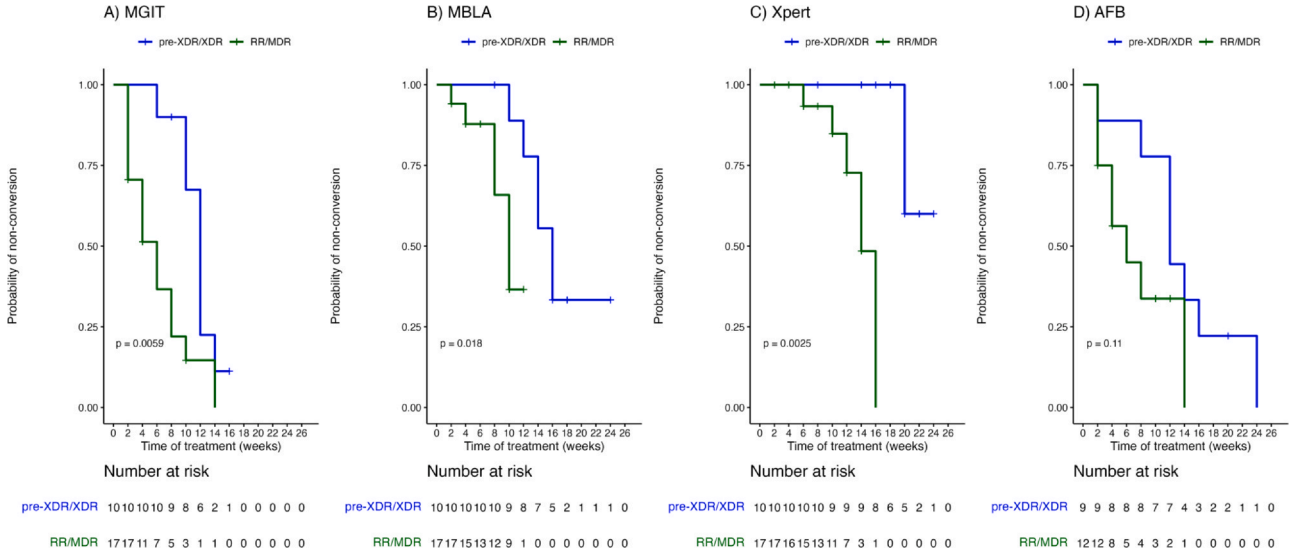

Fig. 7. Probability of sputum positivity during treatment in MDR/RR-TB versus pre-XDR/XDR-TB patients. Analysis included 27 patients for whom sufficiently complete DST- data were available to classify them either as MDR/RR-TB or pre-XDR/XDR-TB patients. Probability of non-conversion over time of treatment is shown for (A) Mycobacteria growth indicator tube (MGIT) (B) Molecular bacterial load assay (MBLA), (C) GeneXpert MTB RIF/ Ultra (Xpert) and (D) acid fast bacilli (AFB). Numbers at risk are shown below. Difference between both groups was tested using log-rank test.

Overall, the MBLA had a superior performance than Xpert measured by higher concordance to MGIT TTP, resulting in both higher sensitivity and specificity.

A major advantage of microscopy in comparison to MGIT, MBLA and Xpert is its low cost, easy performance and, as a result, its worldwide availability.[1] However, for the diagnosis of TB, microscopy has previously been shown to be less sensitive than other diagnostic methods, including the MBLA.[23] Here, a decrease of AFB reflected a treatment response in patients with culture-confirmed TB with slightly higher overall concordance to culture than MBLA. Musisi et al. describe the overall specificity in microscopy to be as good as the MBLA. Also, during the first 2 weeks, test positivity rates of MGIT, MBLA and microscopy were similarly high.[23] However, in our study microscopy did not detect 20.7% of admitted individuals before therapy. Additionally, positivity rate as well as concordance to MGIT were noticeably lower in microscopy than in MBLA until 4 weeks of treatment. One reason for this difference is likely to be attributed to the different LOD of 5 $\times 10^2$ (MBLA; calculated in this study) compared to 1 $\times 10^4$ (microscopy[12,13]) bacteria per ml. Microscopy may therefore miss low numbers of viable bacteria still present in sputum. Overall and when considering the entire course of therapy, the MBLA was the best non-culture-based tool for monitoring concordance with MGIT results at diagnosis and within the first weeks of therapy while microscopy performed better towards the end of the observation period. This supports the use of the MBLA for determining the EBA of medications and monitoring treatment especially in sputum-smear-negative TB patients.[37] This is particularly important in children and PLHIV.[38,39]

The time point at which treatment success should be determined in the therapy of DR-TB is subject of current debate. It is proposed that the bactericidal activity in treatment regimens should not be evaluated at day 14 but later such as day 56 for new MDR/RR-TB regimens.[19] The MBLA had not previously been compared to the other diagnostic procedures at 2-week intervals over the course of therapy in individuals with DR-TB. Our data confirm that culture conversion takes place later in pre-XDR/XDR in comparison to MDR/RR-TB patients and that the probability of non-conversion in MGIT is higher in patients with pre-XDR/XDR than in those with MDR/RR-TB.[39] However, compared to the other diagnostic methods the MBLA predicted treatment success best very early during therapy, irrespective of the level of *M. tuberculosis* drug-resistance. The MBLA demonstrated a significant reduction in bacterial load as early as week 2 and proved effective in predicting delayed culture conversion in patients with pre-XDR/XDR-TB compared to those with MDR/RR-TB.

The present study is the first to monitor TB treatment responses by MBLA at 2-week intervals. However, our analysis had 2 main limitations. First, a larger number of participants, including those failing therapy and experiencing relapse, and a longer observation period would have been necessary to evaluate MBLA for predicting relapse. Second, because MBLA can recognize non-culturable but viable and possibly dormant bacteria in the so-called post-antibiotic lag of growth,[31] a longer follow-up would have been necessary to make a comparative statement regarding relapse of previously non-culturable/dormant bacteria in the present study. This would most likely help to explain the presence of *M. tuberculosis* mRNA in PET-CT positive lesions in sputum culture-negative “cured” TB patients at 12 months follow-up,[38] suggesting a dormant status, in which bacteria have not been completely eradicated but are refractive to culture.[40]

In summary, in contrast to all other diagnostic tests MBLA was able to clearly identify early changes in bacterial load in patients with both, MDR/RR- and pre-XDR/XDR TB. Therefore, MBLA represents a rapid and sensitive biomarker for monitoring treatment success and early identification of unsuccessful culture conversion and therapeutic failure independent of the level of drug resistance.

Funding information

This work was supported by the German Center for Infection Research (DZIF; TI 07.003_Neumann_00 to MN and CH; 02.709 to CL).

Author contributions

JH, CH and BK conceptualized and designed the study. DC, CP, AD, OP, RM conducted the trial, collected sputum samples, performed mycobacterial cultures. IH prepared the internal control. MN and AH performed experiments. MN collected the data. MN, MR, DC and BK analyzed the data. MN, CH and BK wrote the manuscript. IH, JH and CL critically revised the manuscript. All authors approved the manuscript.

Declaration of Competing Interest

J.H. received a honorarium for lectures from Chiesi, Böhringer Ingelheim, and Sanofi, which have nothing to do with the content of this manuscript. C.L. has received an honorarium for consultation service to INSMED, a company that produced liposomal amikacin as an inhalation suspension for the treatment of NTM-PD outside of the scope of this work. He received speakers’ honoraria from INSMED, GSK, Gilead, Astra Zeneca, MedUpdate and MedUpdateEurope outside of the scope of this study. All other authors declare that they have no known competing financial interests or personal relationships that could have appeared to influence the work reported in this paper.

Acknowledgments

The authors would like to thank the patients who participated in this study. The authors thank Moritz Garrelts for advice on the MBLA.

Appendix A. Supporting information

Supplementary data associated with this article can be found in the online version at doi:10.1016/j.jinf.2024.106399.

References

1. WHO. *Global tuberculosis report – 2022*. Geneva, Switzerland: WHO; 2023.
2. Motta I, Boeree M, Chesov D, Dheda K, Gunther G, Horsburgh Jr. CR, et al. *Recent advances in the treatment of tuberculosis. Clin Microbiol Infect* 2023 Jul 22;**30**(9):1107–14. PubMed PMID: 37482332. Epub 20230722.
3. Pedersen OS, Holmgaard FB, Mikkelsen MKD, Lange C, Sotgiu G, Lillebaek T, et al. *Global treatment outcomes of extensively drug-resistant tuberculosis in adults: a systematic review and meta-analysis. J Infect* 2023 Sep;**87**(3):177–89. PubMed PMID: 37356629. Epub 20230623.
4. Lange C, Dheda K, Chesov D, Mandalakas AM, Udwadia Z, Horsburgh Jr. CR. *Management of drug-resistant tuberculosis. Lancet* 2019 Sep 14;**394**(10202):953–66. PubMed PMID: 31526739.
5. Gunther G, Guglielmetti L, Leu C, Lange C, van Leth F, Tuberculosis Network European Trials group. *Availability and costs of medicines for the treatment of tuberculosis in Europe. Clin Microbiol Infect* 2023 Jan;**29**(1):77–84. PubMed PMID: 35961488. Pubmed Central PMCID: PMC9801521. Epub 20220810.
6. Gunther G, Guglielmetti L, Leu C, van Leth F, Lange C. *Relative cost of multidrug-resistant TB medicines in Europe. Int J Tuberc Lung Dis* 2023 May 1;**27**(5):341–4. PubMed PMID: 37143231.
7. (GTP) GTP. Meeting report of the WHO expert consultation on the definition of extensively drug-resistant tuberculosis; 2021.
8. Nyang'wa BT, Berry C, Kazounis E, Motta I, Parpieva N, Tigay Z, et al. *A 24-week, all-oral regimen for rifampin-resistant tuberculosis. N Engl J Med* 2022 Dec 22;**387**(25):2331–43. PubMed PMID: 36546625.
9. Conradie F, Bagdasaryan TR, Borisov S, Howell P, Mikiashvili L, Ngubane N, et al. *Bedaquiline-pretomanid-linezolid regimens for drug-resistant tuberculosis. N Engl J Med* 2022 Sep 1;**387**(9):810–23. PubMed PMID: 36053506. Pubmed Central PMCID: PMC9490302.
10. Suarez I, Funger SM, Kroger S, Rademacher J, Fatkenheuer G, Rybniker J. *The diagnosis and treatment of tuberculosis. Dtsch Arztebl Int* 2019 Oct 25;**116**(43):729–35. PubMed PMID: 31755407.

11. Bloom BR, Atun R, Cohen T, Dye C, Fraser H, Gomez GB, et al. *Tuberculosis.* In: Holmes KK, Bertozzi S, Bloom BR, Jha P, editors. Major Infectious Diseases. 3rd ed. Washington, DC: The International Bank for Reconstruction and Development/ The World Bank; 2017.
12. van der Kuyp F, Mahan CS. *Prolonged positivity of sputum smears with negative cultures during treatment for pulmonary tuberculosis. Int J Tuberc Lung Dis* 2012 Dec;**16**(12):1663–7. PubMed PMID: 23131266. eng.
13. Hobby GL, Holman AP, Iseman MD, Jones JM. *Enumeration of tubercle bacilli in sputum of patients with pulmonary tuberculosis. Antimicrob Agents Chemother* 1973 Aug;**4**(2):94–104. PubMed PMID: 4208508. Pubmed Central PMCID: PMC444512.
14. Cornfield DB, Beavis KG, Greene JA, Bojak M, Bondi J. *Mycobacterial growth and bacterial contamination in the mycobacteria growth indicator tube and BACTEC 460 culture systems. J Clin Microbiol* 1997 Aug;**35**(8):2068–71. PubMed PMID: 9230383. Pubmed Central PMCID: PMC229904.
15. Mukamolova GV, Turapov O, Malkin J, Woltmann G, Barer MR. *Resuscitation-promoting factors reveal an occult population of tubercle Bacilli in Sputum. Am J Respir Crit Care Med* 2010 Jan 15;**181**(2):174–80. PubMed PMID: 19875686. Pubmed Central PMCID: PMC2809243. Epub 20091029.
16. Kayigire XA, Friedrich SO, Venter A, Dawson R, Gillespie SH, Boeree MJ, et al. *Direct comparison of Xpert MTB/RIF assay with liquid and solid mycobacterial culture for quantification of early bactericidal activity. J Clin Microbiol* 2013 Jun;**51**(6):1894–8. PubMed PMID: 23596237. Pubmed Central PMCID: PMC3716052. Epub 20130417.
17. Honeyborne I, McHugh TD, Phillips PP, Bannoo S, Bateson A, Carroll N, et al. *Molecular bacterial load assay, a culture-free biomarker for rapid and accurate quantification of sputum Mycobacterium tuberculosis bacillary load during treatment. J Clin Microbiol* 2011 Nov;**49**(11):3905–11. PubMed PMID: 21900522. Pubmed Central PMCID: 3209113.
18. Jafari C, Olaru ID, Daduna F, Lange C, Kalsdorf B. *Rapid diagnosis of recurrent paucibacillary tuberculosis. Pathog Immun* 2022;**7**(2):189–202. PubMed PMID: 37207169. Pubmed Central PMCID: PMC10189871. Epub 20230419. eng.
19. Mbelele PM, Mpolya EA, Sauli E, Mtafya B, Ntinginya NE, Addo KK, et al. *Mycobactericidal effects of different regimens measured by molecular bacterial load assay among people treated for multidrug-resistant tuberculosis in Tanzania. J Clin Microbiol* 2021 Mar 19;**59**(4):e02927-20. PubMed PMID: 33536294. Pubmed Central PMCID: PMC8092737. Epub 20210319.
20. Theron G, Venter R, Calligaro G, Smith L, Limberis J, Meldau R, et al. *Xpert MTB/RIF results in patients with previous tuberculosis: can we distinguish true from false positive results? Clin Infect Dis* 2016 Apr 15;**62**(8):995–1001. PubMed PMID: 26908793. Pubmed Central PMCID: PMC4803105. Epub 20160216. eng.
21. Friedrich SO, Rachow A, Saathoff E, Singh K, Mangu CD, Dawson R, et al. *Assessment of the sensitivity and specificity of Xpert MTB/RIF assay as an early sputum biomarker of response to tuberculosis treatment. Lancet Respir Med* 2013 Aug;**1**(6):462–70. PubMed PMID: 24429244. Epub 20130701.
22. Honeyborne I, Mtafya B, Phillips PP, Hoelscher M, Ntinginya EN, Kohlenberg A, et al. *The molecular bacterial load assay replaces solid culture for measuring early bactericidal response to antituberculosis treatment. J Clin Microbiol* 2014 Aug;**52**(8):3064–7. PubMed PMID: 24871215. Pubmed Central PMCID: 4136187.
23. Musisi E, Wamutu S, Ssengooba W, Kasiinga S, Sessolo A, Sanyu I, et al. *Accuracy of the tuberculosis molecular bacterial load assay to diagnose and monitor response to anti-tuberculosis therapy: a longitudinal comparative study with standard-of-care smear microscopy, Xpert MTB/RIF Ultra, and culture in Uganda. Lancet Microbe* 2024;**5**(4):e345–54.
24. Bossuyt, Reitsma PM, Bruns DE JB, Gatsonis CA, Glasziou PP, Irwig L, et al. *STARD 2015: an updated list of essential items for reporting diagnostic accuracy studies. BMJ* 2015 Oct 28;**351**:h5527. PubMed PMID: 26511519. Pubmed Central PMCID: PMC4623764. Epub 20151028. eng.
25. Cristina AO. *GHID Metodologic de implementare a Programului national de prevenire, supraveghere si control al tuberculozei.* Bukarest, Romania: Ministerul Sanatatii; 2015.
26. Lump R, Van Deun A, Bastian I, Fitz-Gerald M, editors. Laboratory Diagnosis of Tuberculosis by Sputum Microscopy. Adelaide, Australia: SA Pathology; 2013.
27. Satapathy P, Das D, Murmu B, Kar S. *Decontamination of sputum for longer time in sodium hydroxide for isolation of Mycobacterium tuberculosis. Int J Mycobacteriology* 2014;**3**(4):290–2.
28. Chakravorty S, Simmons AM, Rowneki M, Parmar H, Cao Y, Ryan J, et al. *The New Xpert MTB/RIF Ultra: improving detection of mycobacterium tuberculosis and resistance to rifampin in an assay suitable for point-of-care testing. mBio* 2017 Aug 29;**8**(4):e00812-17. PubMed PMID: 28851844. Pubmed Central PMCID: PMC5574709. Epub 20170829. eng.
29. WHO. *Xpert MTB/RIF Implementation Manual: Technical and Operational 'How-To'; Practical Considerations.* WHO; 2014.
30. Team RC. R: A Language and Environment for Statistical Computing Vienna: R Foundation for Statistical Computing; 2023.
31. Ntinginya NE, Bakuli A, Mapamba D, Sabiiti W, Kibiki G, Minja LT, et al. *Tuberculosis molecular bacterial load assay reveals early delayed bacterial killing in patients with relapse. Clin Infect Dis* 2023 Feb 8;**76**(3):e990–4. PubMed PMID: 35717643. Pubmed Central PMCID: PMC9907486. eng.
32. Ntinginya NE, Bakuli A, Mapamba D, Sabiiti W, Kibiki G, Minja LT, et al. *Tuberculosis molecular bacterial load assay reveals early delayed bacterial killing in patients with relapse. Clin Infect Dis* 2023 Feb 8;**76**(3):e990–4. PubMed PMID: 35717643. Pubmed Central PMCID: PMC9907486.
33. Sabiiti W, Azam K, Farmer ECW, Kuchaka D, Mtafya B, Bowness R, et al. *Tuberculosis bacillary load, an early marker of disease severity: the utility of tuberculosis Molecular Bacterial Load Assay. Thorax* 2020 Jul;**75**(7):606–8. PubMed PMID: 32354738. Pubmed Central PMCID: PMC7361026. Epub 20200430.
34. Hai HT, Vinh DN, Thu DDA, Hanh NT, Phu NH, Srinivasan V, et al. *Comparison of the Mycobacterium tuberculosis molecular bacterial load assay, microscopy and GeneXpert versus liquid culture for viable bacterial load quantification before and after starting pulmonary tuberculosis treatment. Tuberculosis* 2019 Sep 21;**119**:101864. PubMed PMID: 31568973. Epub 2019/10/01.
35. Kana BD, Gordhan BG, Downing KJ, Sung N, Vostroktunova G, Machowski EE, et al. *The resuscitation-promoting factors of Mycobacterium tuberculosis are required for virulence and resuscitation from dormancy but are collectively dispensable for growth in vitro. Mol Microbiol* 2008 Feb;**67**(3):672–84. PubMed PMID: 18186793. Pubmed Central PMCID: PMC2229633. eng.
36. Stewart GR, Robertson BD, Young DB. *Tuberculosis: a problem with persistence. Nat Rev Microbiol* 2003 Nov;**1**(2):97–105. PubMed PMID: 15035039. eng.
37. Gillespie SH, DiNardo AR, Georghiou SB, Sabiiti W, Kohli M, Panzner U, et al. *Developing biomarker assays to accelerate tuberculosis drug development: defining target product profiles. Lancet Microbe* 2024;**5**:100869.
38. Malherbe ST, Shenai S, Ronacher K, Loxton AG, Dolganov G, Kriel M, et al. *Persisting positron emission tomography lesion activity and Mycobacterium tuberculosis mRNA after tuberculosis cure. Nat Med* 2016 Oct;**22**(10):1094–100. PubMed PMID: 27595324. Pubmed Central PMCID: PMC5053881. Epub 20160905.
39. Ncha R, Variava E, Otwombe K, Kawonga M, Martinson NA. *Predictors of time to sputum culture conversion in multi-drug-resistant tuberculosis and extensively drug-resistant tuberculosis in patients at Tshepong-Klerksdorp Hospital. S Afr J Infect Dis* 2019;**34**(1):111. PubMed PMID: 34485452. Pubmed Central PMCID: PMC8377786. Epub 20190826.
40. Mtafya B, Sabi I, John J, Sichone E, Olomi W, Gillespie SH, et al. *Systematic assessment of clinical and bacteriological markers for tuberculosis reveals discordance and inaccuracy of symptom-based diagnosis for treatment response monitoring. Front Med* 2022;**9**:992451. PubMed PMID: 36419786. Pubmed Central PMCID: PMC9677322. Epub 20221028.