Model-Based Relationship between the Molecular Bacterial Load Assay and Time to Positivity in Liquid Culture

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ABSTRACT The molecular bacterial load (MBL) assay is a new tuberculosis biomarker which provides results in ~4 hours. The relationship between MBL and time-to-positivity (TTP) has not been thoroughly studied, and predictive models do not exist. We aimed to develop a model for MBL and identify the MBL-TTP relationship in patients. The model was developed on data from 105 tuberculosis patients from Malawi, Mozambique, and Tanzania with joint MBL and TTP observations quantified from patient sputum collected for 12 weeks. MBL was quantified using PCR of mycobacterial RNA and TTP using the mycobacterial growth indicator tube (MGIT) 960 system. Treatment consisted of isoniazid, pyrazinamide, and ethambutol in standard doses together with rifampin 10 or 35 mg/kg of body weight. The developed MBL-TTP model included several linked submodels, a component describing decline of bacterial load in sputum, another component describing growth in liquid culture, and a hazard model translating bacterial growth into a TTP signal. Additional components for contaminated and negative TTP samples were included. Visual predictive checks performed using the developed model gave good description of the observed data. The model predicted greater total sample loss for TTP than MBL due to contamination and negative samples. The model detected an increase in bacterial killing for 35 versus 10 mg/kg rifampin ($P = 0.002$). In conclusion, a combined model for MBL and TTP was developed that described the MBL-TTP relationship. The full MBL-TTP model or each submodel was used separately. Second, the model can be used to predict biomarker response for MBL given TTP data or *vice versa* in historical or future trials.

KEYWORDS *Mycobacterium tuberculosis*, biomarker, mathematical modeling, pharmacodynamics, pharmacometrics, tuberculosis

The tuberculosis (TB) burden in patients is usually quantified by culture on solid medium or in liquid culture, such as the mycobacterial growth indicator tube (MGIT) (1). In the diagnostic phase, the TB burden quantification gives information on disease severity, and when collected during treatment, it gives information on treatment response. Quantification has usually been done using CFUs on solid medium (2) or time-to-positivity (TTP) in liquid culture using the MGIT system (1).

MGIT TTP has advantages over CFU counts on solid medium by being less labor intensive and more sensitive (3), but like CFU, TTP is hampered by a high degree of sample loss due to contamination and the long time taken before results are available (5 to 42 days) (4). This delay has a particular impact when quantitative methods are

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TABLE 1 Baseline patient characteristics for all data and by study site

Parameter	Values by site			
	All (n = 105)	Malawi (n = 20)	Mozambique (n = 53)	Tanzania (n = 32)
Weight (kg) ^a	54 (37–74)	55.8 (43–70)	54.0 (37–74)	
Male sex (n [%])	77 (73)	14 (70)	35 (66)	28 (88)
HIV positive (n [%])	45 (42.9)	9 (45.0)	36 (67.9)	0 (0)
Rifampin (10 mg/kg) (n [%])	93 (89)	20 (100)	53 (100)	20 (63)
Rifampin (35 mg/kg) (n [%])	12 (11)	0 (0)	0 (0)	12 (38)
Baseline TTP (days) ^a	4.22 (1.02–23.1)	6.02 (3.14–16.10)	4.13 (2.00–9.60)	3.21 (1.02–23.1)
Negative baseline TTP (n [%]) ^b	2 (1.90)	0 (0)	2 (3.77)	0 (0)
Baseline MBL (log ₁₀ CFU/ml) ^a	5.91 (2.62–8.37)	5.43 (3.61–7.83)	6.35 (2.62–8.28)	5.26 (3.04–8.37)
Negative baseline MBL (n [%]) ^c	1 (0.95)	0 (0)	1 (1.89)	0 (0)

^aData are median values (ranges). Abbreviations: TTP, time-to-positivity; MBL, molecular bacterial load.

^bDefined as a TTP longer than 42 days.

^cDefined as a log₁₀ MBL value below 2.

used in patient care where individual treatment adjustment decisions based on bacterial response should ideally be quick. Time-to-positivity is a time-to-event variable representing an indirect measurement of the bacterial load (high CFU gives short TTP).

The molecular bacterial load (MBL) assay is a new TB biomarker which is fast (~4 hours) (4) and has limited risk of contamination (5). This is because MBL is a non-culture-based real-time PCR method relying on reverse transcription-quantitative PCR (RT-qPCR) of 16S rRNA to quantify bacterial load (6). Viable TB cells contain 16S rRNA which makes MBL a continuous measurement of bacterial load. MBL can be used to predict bacterial load.

MBL has weak to moderate correlation with TTP in clinical trials with reported correlations of -0.5 (4) and -0.8 (7) using Spearman rank correlation. The weak correlation is not surprising since these biomarkers are different, with MBL being a direct, continuous variable and TTP being an indirect, time-to-event variable. Nonlinear mixed effect models have been applied separately to MBL (5) and TTP (8–10) data sets but a combined MBL-TTP model has not been presented previously. The current TTP-only models do not consider contaminated samples which are a common occurrence in culture-based detection of TB.

Identifying the link between MBL and TTP could contribute to the understanding of the difference in how these biomarkers quantify bacterial burden. A combined MBL-TTP model could also be used to predict one biomarker response given information about the other biomarker, providing additional insights from historical trials. Given that in some studies contamination is common especially later in treatment, including a component for contaminated TTP samples is warranted.

The objectives of this study were to develop a model for MBL and identify the relationship between MBL and TTP in pulmonary TB patients by constructing a joint MBL-TTP model.

RESULTS

Patient data. Patient baseline characteristics are summarized in Table 1. For MBL, 851 samples were analyzed, of which 277 samples (32.5%) were below the employed lower limit of quantification (LLOQ) of 100 CFU/ml. For TTP, 659 samples were analyzed, of which 192 samples (29.1%) were contaminated and 90 samples (13.7%) were negative (i.e., the TTP was greater than 42 days).

Submodel for MBL data. The developed sputum model included two mycobacterial subpopulations, namely, B1 and B2, where the treatment had exponential killing of both subpopulations where the MBL prediction was assumed to represent the total bacterial population [i.e., $MBL(t_i) = B1_s(t_i) + B2_s(t_i)$]. The B1 subpopulation had greater abundancy (~99%) than B2 (~1%) at pretreatment, and the B1 subpopulation was also more easily killed than B2 (B1 killed ~3.5 times more rapidly than B2). Thus, B2 was more tolerant to treatment-induced bacterial killing which led to B2 becoming more abundant than B1 during late treatment. A statistically significant increased kill (1.66-

FIG 1 Schematic representation of the final combined MBL-TTP model. The treatment is represented by an “on/off” effect, i.e., the drugs kill the bacteria in the presence of drugs. The killing of bacteria, represented by two mycobacterial subpopulations in the sputum ($B1_s$ and $B2_s$) is governed by two first-order kill-rates (k_1 and k_2) in the sputum model. The sum of $B1_s$ and $B2_s$ constitutes the MBL submodel and gives the prediction of MBL (MBL model, lowest middle box). The predicted bacterial densities of the subpopulations in sputum ($B1_s$ and $B2_s$) are the inoculum for the mycobacterial growth model (top middle box), at the corresponding sampling time point. Only $B1_c$ in the liquid culture has the ability to grow. The $B2_c$ cannot grow but can transfer into the $B1_c$ subpopulation with a first-order rate of k_{21} . A TTP model (top right box) translates the growth within the mycobacterial growth model to a positive TTP signal where only $B1_c$ contributes to the probability of a positive TTP signal. This is done using survival modeling, where $B1_c$ multiplied by a time-varying factor accounts for the relative contribution of each $B1_c$ bacterial cell to the probability of a positive signal. Negative TTP samples are described by a nonlinear (E_{max}) relationship between the bacterial density in sputum ($B1_s + B2_s$) and probability of a negative TTP sample (upper middle box). Contaminated TTP samples are described by a function for the probability of a contaminated sample that increase linearly with time on treatment (lower middle box). Abbreviations: k_1 , first-order bacterial kill rate of bacterial subpopulation $B1_s$; k_2 , first-order bacterial kill rate of bacterial subpopulation $B2_s$; MBL, molecular bacterial load; TTP, time-to-positivity.

fold) of $B1$ (but not $B2$) by rifampin 35 versus 10 mg/kg ($P = 0.002$) was included in the model. The HIV covariate on initial bacterial load was not statistically significant. The MBL model gave a good description of the observed data according to a visual predictive check (VPC) (see Fig. S1 in the supplemental material).

Inclusion of two mycobacterial subpopulations in the sputum model gave a significantly better fit to the observed data than a sputum model only, including a single subpopulation ($P < 0.00001$). The treatment had first-order killing of both subpopulations (included as an “on/off” treatment effect). In the final MBL sputum submodel, bacteria were assumed to be unable to grow or transfer between subpopulations in sputum. A three-subpopulation model was not supported by the data as it resulted in an unstable estimation.

Inclusion of interindividual variability in initial bacterial load of both subpopulations ($B1$ and $B2$), also including a correlation between the subpopulations, led to a significantly better fit to the observed data and was therefore included in the final model.

The final model for MBL with an intended use of modeling future MBL-only data sets, referred to as the standalone MBL model, is given in Data Set S2 in the supplemental material. Parameter estimates are shown in Data Set S3 in the supplemental material.

Combined MBL-TTP model. The structure of the final combined MBL-TTP model is shown in Fig. 1. The dynamics of each submodel are shown for baseline and week 12 samples in addition to week 4 (which was considered relevant since it is located in the transition between the initial rapid decline and the later slower decline for bacterial load in sputum) for a typical individual in Fig. 2. The final combined MBL-TTP model included the same sputum model as described above for the standalone MBL model.

FIG 2 Typical model predictions within each component of the final combined MBL-TTP model for a patient treated with isoniazid, pyrazinamide, ethambutol, and 10 mg/kg rifampin. (a) Shows predictions from the sputum model where the line represents the prediction of MBL. (b) Shows predictions of the bacterial density in the liquid culture. Remaining panels show the predicted time-varying probability of not having a positive TTP signal (c), probability of contaminated TTP samples (d), and probability of negative TTP samples (e). The different symbols in (a, d, and e) show the dynamics within the corresponding submodel where circles represent an early baseline sample, squares represent an intermediate (4-week) sample, and triangles represent a late (week 12) sample. In (b and c), the circles represent an early (baseline) sample, the squares represent an intermediate (4-week) sample and the triangles represent a late (week 12) sample. Abbreviations: MBL, molecular bacterial load; TTP, time-to-positivity.

The same subpopulations as described within the sputum model existed within the mycobacterial growth model where only the B1 population could grow. However, B2 bacteria were able to transfer into B1 in the liquid culture (Fig. 1). The model component for contaminated TTP samples included a linear relationship between time on treatment and risk of contamination. Finally, the model included a component for negative TTP, including a nonlinear maximum effect ($E_{\max}$) relationship between bacterial load in sputum and probability of negative TTP. Fig. 3 compares sample loss due to negative and/or contaminated samples for TTP versus MBL. The figure shows that both MBL and TTP have a similar degree of negative samples (Fig. 3a), but due to the

FIG 3 Sample loss for MBL (filled circles) and TTP (filled triangles) due to (a) negative samples, i.e., assuming no loss of samples due to contamination; (b) contaminated samples, assuming no loss of samples due to negativity; and (c) total sample loss reflecting the combined sample loss due to negative samples and contaminated samples. Abbreviations: TTP, time-to-positivity; MBL, molecular bacterial load.

FIG 4 The relationship between TTP and MBL. The VPC shows the median (solid line) and 5th and 95th percentiles (lower and upper dashed lines, respectively) of observed data compared with the corresponding percentiles of simulated data (shaded areas) based on 1,000 simulated data sets where lighter gray are percentiles and darker gray is median. The open circles show the actual observations. Abbreviations: TTP, time-to-positivity; MBL, molecular bacterial load; VPC, visual predictive check.

much higher contamination of TTP (Fig. 3b), the MBL assay gives more information in terms of noncontaminated, positive samples (Fig. 3c).

Simulated data from the final combined MBL-TTP model gave a good description of the observed data which showed that the model was appropriate given the data. A plot of observed and model-predicted TTP versus MBL shows that the final model accurately described the observed pattern between the biomarkers (Fig. 4). A VPC of MBL versus time (Fig. 5) and a Kaplan-Meier VPC of TTP versus time in liquid culture for different treatment weeks (Fig. 6) also showed that the model described the observed data well. Parameter estimates of the final combined MBL-TTP model are shown in Table 2. Precision looked fine for all parameters. All parameters were estimated on a linear scale.

For the mycobacterial growth model, only B1 could grow but B2 could transfer into B1. A transfer rate parameter (k_{21}) described the transfer between B2 and B1 and was set to the same value as the growth rate (k_G). Estimation of a unique k_{21} led to an unstable model and was not statistically significant ($P = 0.176$). The growth function that best described the growth of B1 was found to be the Gompertz model.

Bacterial growth was linked to the probability of a positive TTP signal using a time-to-event approach where only B1 contributed to the probability (hazard) of a positive signal (since B2 was nongrowing B2 does not contribute directly to hazard). The contribution of each B1 bacterium to the probability of a positive signal was determined by a scaling parameter. The scaling parameter was time-varying in the final model where the value decreased exponentially from a baseline value down to a steady-state value. Having a similar time-varying component for other potentially relevant parameters, such as the growth rate (k_G) or introducing a lag-time for growth, did not lead to a stable model.

The model for contaminated samples was different between sites. However, parameters estimated from the Tanzania site were considered the most appropriate model. For Tanzania, the observed contamination rate was low initially ($\sim 10\%$) and increased linearly to reach a contamination rate of $\sim 60\%$ by week 12. For Malawi, contamination was not determined (i.e., no blood agar test was done). For Mozambique, contamination was moderately high (~ 30 to 40%) across all time points. A VPC for contamination versus time (see Fig. S2 in the supplemental material) confirmed that the model gave a good description of the observed contamination data.

A submodel was included in the final model to handle negative TTP. The probability of a negative sample increased as bacterial load in sputum decreased (10). An inhibitory sigmoidal $E_{\max}$ model described the relationship where the lowest possible probability

FIG 5 Visual predictive check (VPC) for the MBL data using the final combined MBL-TTP model. The VPC shows the median (solid line) and 5th and 95th percentiles (lower and upper dashed lines, respectively) of observed data compared with the corresponding percentiles of simulated data (shaded areas) where lighter gray are percentiles and darker gray is median. The open circles show the actual observations and the dotted line is the lower limit of quantification for MBL. The shaded areas represent 95% confidence interval of simulated data based on 1000 simulated data sets. Abbreviations: MBL, molecular bacterial load.

of a negative sample was estimated to be 3.3% and occurred at a very high bacterial density. The probability of a negative sample was half-maximal at a bacterial density of 48.8 CFU/ml. This is a rather low number which represents roughly half the LLOQ of 100 CFU/ml which was used in this analysis for MBL. A model where negative TTP samples were handled using right-censoring within the hazard model (which is common practice for time-to-event models) did not lead to an acceptable description of the observed data and was, therefore, discarded.

Interindividual variability was included for the scaling parameter that accounted for the contribution of B1 to the probability of a positive signaling event in the liquid culture.

Utility of the final model. It was possible to reestimate the final combined MBL-TTP model using only MBL data if the TTP-related parameters were fixed to the parameters of the final model, where the model gave good description of the observed MBL data (see Fig. S3 in the supplemental material). The final model with the TTP-related parameters fixed can be found in Data Set S2. Likewise, we successfully reestimated the final model with only TTP data with MBL-related parameters fixed to the final model estimates with good fit to the observed data (see Fig. S4 in the supplemental material). The model estimated with TTP-only data can be used as a standalone TTP model to estimate TTP-only data sets. The final MBL-TTP model with the MBL-related parameters fixed can be found in Data Set S2.

A comparison of parameter estimates for the final combined MBL-TTP model estimated including all data, the final model reestimated with MBL or TTP data only, and the standalone MBL model can be found in Data Set S3. There was consistency in the estimated parameters between all the models. The covariate effect of enhanced

FIG 6 Kaplan-Meier visual predictive check (VPC) of the TTP data using the final combined MBL-TTP model. The VPC shows the observed Kaplan-Meier (survival) curve for each weeks' TTP as solid lines and the simulated Kaplan-Meier 95% confidence intervals are shown as blue shaded areas ($n = 1,000$ simulated data sets). Abbreviations: TTP, time-to-positivity; MGIT, mycobacterial growth incubator tube.

TABLE 2 Parameter estimates of the final combined MBL-TTP model^{a,b}

Parameter by model type ^c	Description	Estimate
Sputum		
$B_{1,0,s}$ (CFU/ml)	Initial bacterial load of mycobacterial subpopulation 1 in sputum	0.365×10^6 (28.8)
k_1 (week ⁻¹)	First-order bacterial kill rate of subpopulation 1 in sputum	1.71 (8.70)
$B_{2,0,s}$ (CFU/ml)	Initial bacterial load of mycobacterial subpopulation 2 in sputum	0.00430×10^6 (51.4)
k_2 (week ⁻¹)	First-order bacterial kill rate of subpopulation 2 in sputum	0.494 (9.50)
Dose _{35mg}	Fold increase in bacterial killing of k_1 by 35 versus 10 mg/kg rifampin	1.66 (14.0)
IIV $B_{1,0,s}$ (%)	Interindividual variability in $B_{1,0,s}$	239 (9.18)
IIV $B_{2,0,s}$ (%)	Interindividual variability in $B_{2,0,s}$	227 (10.9)
Corr $B_{1,0,s} - B_{2,0,s}$ (%)	Correlation between $B_{1,0,s}$ and $B_{2,0,s}$	45.4 (15.6)
ε (%)	Additive error on log scale for MBL data	79.7 (4.96)
Mycobacterial growth		
k_G (day ⁻¹) ^d	Mycobacterial growth of subpopulation 1 in liquid culture	0.395 (8.50)
k_{21} (day ⁻¹) ^d	Transfer rate from subpopulation 2 to subpopulation 1 in liquid culture	0.395 (8.50)
$B_{\max}$ (CFU/ml)	Maximal bacterial load in liquid culture	166×10^6 (24)
TTP		
Scale _{BL}	Baseline value of scaling parameter accounting for the contribution of subpopulation 1 bacteria to the probability of a positive TTP signal	6.68×10^{-9} (31.6)
IIV Scale _{BL} (%)	Interindividual variability in scale _{BL}	80.6 (18.1)
Scale _{SS}	Steady-state value of scaling parameter	0.601×10^{-9} (24.3)
k_5 (week ⁻¹)	First-order rate constant for time-varying change of the scaling parameter	1.28 (25.1)
For negative TTP samples		
$p_{\max}$	Maximal probability of a positive TTP sample	0.967 (1.50)
B_{50}	Bacterial load of subpopulation 1 and subpopulation 2 in sputum at which the probability of a positive TTP value is half maximal	49.8 (33.7)
γ	Gamma-factor for nonlinear $E_{\max}$ relationship for negative TTP samples	0.756 (18.9)
For contaminated TTP samples		
$p_{\text{con,base}}$ ^e	Baseline probability of a contaminated TTP sample	0.0910 (39.0)
k_p ^c	Linear time-varying increase of probability of a contaminated TTP sample	0.0416 (13.9)

^aThe reported values are the final estimates with relative standard error (RSE) shown in parentheses as the approximate coefficient of variation (%CV) on standard deviation scale. The IIV and residual error are shown as the approximate %CV on standard deviation scale, which is calculated using a simple square root formula, namely, %CV = $100 \times \sqrt{\text{variance}}$. Correlation (Corr $B_{1,0,s} - B_{2,0,s}$) is reported as the percentage correlation coefficient. The mathematical structures for the final model were (final NONMEM code in Data Set S2) $B_{1,s}(t_i) = B_{1,0,s} \times e^{-k_1 \times t_i \times \text{dose}_{35\text{mg}}}$ (B1 subpopulation in sputum) and $B_{2,s}(t_i) = B_{2,0,s} \times e^{-k_2 \times t_i}$ (B2 subpopulation in sputum), where dose_{35mg} is 0 for 10 mg/kg rifampin and the individually predicted MBL = $B_{1,s}(t_i) + B_{2,s}(t_i)$; $(dB_{1,c}/dt_c) = B_{1,c}(t_c) \times k_G \times \log[B_{\max}/(B_{1,c}(t_c) + B_{2,c}(t_c))]$ + $k_{21} \times B_{2,c}(t_c)$ (B1 subpopulation in liquid culture) $(dB_{2,c}/dt_c) = -k_{21} \times B_{2,c}(t_c)$ (B2 subpopulation in liquid culture), where the initial conditions for each TTP sample were $B_{1,c}(t_c = 0) = B_{1,s}(t_i = \text{sampling time point})$ and $B_{2,c}(t_c = 0) = B_{2,s}(t_i = \text{sampling time point})$ for the B1 and B2 subpopulations, respectively;

$h(t_c) = B_{1,c}(t_c) \times [\text{scale}_{\text{BL}} + (\text{scale}_{\text{SS}} - \text{scale}_{\text{BL}}) \times (1 - e^{-k_5 \times t_i})]$ (TTP model), where the cumulative hazard $H(t_c) = \int_0^{t_c} h(t_c) dt$ was used to calculate the survival $S(t_c) = e^{-H(t_c)}$; $p_{\text{contaminated,TTP}} = p_{\text{con,base}} + k_{\text{con}} \times t_i$ (model for contaminated TTP samples); and $p_{\text{negative,TTP}} = 1 - p_{\max} \times [B_{1,s}(t_i) + B_{2,s}(t_i)]^\gamma / \{B_{50}^\gamma + [B_{1,s}(t_i) + B_{2,s}(t_i)]^\gamma\}$ (model for negative TTP samples).

^bAbbreviations: TTP, time-to-positivity; t_i , time on treatment; MBL, molecular bacterial load; t_c , time in liquid culture; $p_{\text{contaminated,TTP}}$, probability for a contaminated TTP sample; $p_{\text{negative,TTP}}$, probability for a negative TTP sample.

^cFinal parameter estimates of the MBL standalone model, the model predicting TTP based on MBL, and the model predicting MBL based on TTP are available in Data Set S3.

^d k_{21} and k_G were modeled as a single parameter in the model.

^eThe parameters for contaminated TTP samples were estimated on data from Tanzania, and the other sites included time-constant probabilities of 0 for Malawi (fixed value, since contamination was not measured) and 0.336 (10.0% RSE) for Mozambique.

performance of 35 mg/kg rifampin was estimable using all models, i.e., using MBL or TTP data only as well as with all data.

DISCUSSION

This analysis describes the development of a pharmacometric model to identify the relationship between two critical measures of viable count, namely, MBL and TTP based on data collected during 12 weeks in drug-susceptible TB patients treated with the standard drug combination. In this model, the relationship between the biomarkers was identified successfully.

To make an effective model, it was necessary to include components that described the different data types; MBL is a continuous variable, whereas TTP is a time-to-event variable indirectly reflecting bacterial load. The best sputum model (describing the underlying bacterial load in sputum) was achieved when we included two mycobac-

terial subpopulations (B1 and B2) with treatment, resulting in an exponential fall in viable count for both. The predicted MBL was assumed to be the total bacterial population in sputum [i.e., $MBL(t_i) = B1_s(t_i) + B2_s(t_i)$]. Although the drug effect was included as an “on/off” treatment effect which represents a limitation of the present study, in the future it can/will be replaced by exposure-response relationships in later analyses. In our model, the B1 subpopulation was more abundant than B2 at pretreatment, whereas B2 became more abundant than B1 on late treatment days since B1 was killed more rapidly than B2. This is similar to the report of Honeyborne et al. (5) although their work only included MBL data. We agree with their analysis that the B2 population may represent persisters (5). A three-subpopulation model was tested during the model development. A three-subpopulation model reflective of multiplying, semidormant, and persister cells would have been a more mechanistically plausible structure than the two subpopulations described in this work, as TB is known to exist in at least three subpopulations (11). To interpret these results, we may need to consider that the B1 and B2 subpopulations may also partly contain semidormant cells, although to what extent this occurs is unknown. This also had implications when exploring the relationship between MBL and TTP; with only two subpopulations included we were not able to appropriately explore our hypothesis that MBL reflects more bacterial subpopulations than TTP (i.e., we could not explore if TTP quantified semidormant but not persister cells without semidormant cells in the model). According to the final model structure, both subpopulations contributed to MBL and TTP which can be interpreted as that both biomarkers reflect the same subpopulations. However, we do not have this view of our results, as we still hypothesize that MBL may reflect more subpopulations than TTP and that our results just confirm that there is a large overlap in what subpopulations each biomarker captures. Yet, we found that the three-subpopulation model was not stable, although the reasons for this are unknown.

However, one potential explanation to the instability is that the clinical data used for this analysis contained a suboptimal number of “critical” data points where persisters are expected to be the dominating subpopulation which we believe occur primarily at late time points (Fig. 3). If a lower MBL LLOQ than 100 CFU/ml is applied in a future analysis, it may lead to more critical data points. Another option where critical persister-dominated data points can be studied in controlled settings could be *in vitro* systems. Alternatively, the MBL information can be supplemented with information from staining-based techniques to identify phenotypic resistance based on lipid bodies (12), a study that is under way.

An important advance in this model is the way in which it includes a submodel that allowed us to predict TTP in a mechanistically plausible manner (Fig. 1). The sputum model acted as the fundamental hub within the model where changes in the predicted bacterial load in sputum affected both the resulting MBL and TTP predictions. As anticipated, the relationship between MBL and TTP lies in the sputum model. The most essential way that the sputum model affected the TTP predictions was through the mycobacterial growth model describing growth in liquid culture as well as the hazard model which described how growth affected the probability of a positive signal. This way of linking submodels has been described in other time-to-event models only describing TTP data (8–10). Although the general structure of our model is similar to previous reports, what makes our model unique is the description of two distinct bacterial subpopulations both in the sputum model and in the mycobacterial growth model. The underlying study had no experimental data which could distinguish between the two populations; this was instead described by the mathematical model. In the liquid culture, B2 was nongrowing but could indirectly contribute to growth by transferring into B1, potentially reflecting a shift to a more metabolically active state triggered by the nutrient-rich liquid culture medium. The transfer rate of B1 transferring to B2 ($k_{2,1}$) was set to the same value as the bacterial growth rate (k_G). This was reasonable given the insufficient data to inform differences in these parameters. Furthermore, when the mycobacterial growth model was linked to the hazard model, which translates the growth in the liquid culture to a probability of a positive signal,

only B1 contributed to the probability of a positive TTP. As the underlying reason for a positive signaling is carbon dioxide production, this implies that nongrowing B2 bacteria do not contribute measurably to carbon dioxide production. Both findings, i.e., that B2 is nongrowing and does not produce carbon dioxide, were driven by the data and are important observations. It may explain a disproportionally greater TTP prolongation on early versus late treatment days, as not only did the MGIT inoculum decrease each week but also the proportion of bacteria that can grow and readily produce carbon dioxide immediately upon liquid culture inoculation has also decreased (i.e., the B1/B2 ratio decrease with treatment time). This observation agrees with and provides further insight into a hypothesis generated in a non-model-based analysis comparing CFU, time to appearance of CFU, and TTP (13). In that study (13) there was significant correlation between time to appearance of the first CFU colony on solid media and TTP, suggesting that the fastest growing bacteria have a disproportionally larger contribution to the carbon dioxide production in liquid culture, i.e., a similar interpretation as can be drawn from our work. Another finding within our model that also contributes to this relationship is the time-varying scaling parameter which decreased with time on treatment. The time-varying scaling parameter is, once again, a data-driven finding. Future *in vitro* work should explore the biological explanation.

Previous models have treated TTP as a continuous variable (14–17). Our work suggests that this is not the optimal way to handle these data, as time-to-positivity reflects time-to-event data. As was the case for a previous publication, a time-to-event analysis of TTP revealed an exposure-response relationship of rifampin (8) that was undetected for the same data set when the TTP data were treated as continuous data (18).

One of the challenges of modeling data from TB clinical trials is that previously published MGIT-TTP models lack components for contamination, which is a significant confounder of this assay. Thus, the contamination submodel is a significant improvement on the previously published models for TTP since it allows for real-world clinical trial simulations. Our model can be used to make simulations prior to performing clinical TTP studies to predict the degree of TTP sample loss. We regard the predicted and observed degrees of contamination as high (Fig. 3), suggesting that TTP can be unreliable and difficult to interpret, especially during late treatment; thus it is meaningful to get a reliable expectation on the degree of sample loss. Significantly, it means that MBL, which is not affected by contamination, is a significant improvement over TTP, as shown in Fig. 3. The typical patient is expected to have greater sample loss for TTP than MBL when accounting for both contamination and negative samples. The included component for contaminated TTP was based on time on treatment and was site dependent, but we recommend the contamination model derived from Tanzania for performing clinical trial simulations since contamination for Tanzania data started low at baseline and increased with time on treatment, which represents the most plausible contamination pattern. It has been shown that during early treatment patients produce sputum of better quality than later treatment where patients get healthier, which is associated with a relative inability to produce sputum (19).

A submodel was included to describe negative TTP samples which predicted that lower bacterial densities in sputum gave a higher probability of a negative TTP sample. This way of handling negative TTP samples is similar as a previous model for TTP (10). An $E_{\max}$ model described this relationship (Fig. 2e) and predicted a probability of a negative TTP of 3.3% at very high bacterial densities, suggesting that a fraction of TTP samples will always be negative. The model by Svensson and Karlsson (10) predicted that 3.1% will always be negative, which is similar to our value.

The developed model gave good fit to the observed data according to the diagnostic plots in Fig. 4 and 6. In addition, the parameter precision in the parameters was overall low (Table 2). This shows that from a technical model validation perspective, the presented model is valid.

In this work, we identified a statistically significant increased (1.66-fold) killing effect for 35 versus 10 mg/kg rifampin which indicates that the joint collection of MBL and TTP data used along with our modeling approach is a powerful strategy for detecting

interregimen differences for phase IIb trials. If studies are designed and analyzed according to our approach, phase IIb trial performance may be simplified and could require fewer patients to be recruited. However, this finding was based on data from 12 patients in the dataset that received 35 mg/kg rifampin and the model as such was not tested on any external data.

The utility analysis showed that the model can be used to analyze MBL data alone to predict TTP and *vice versa* if parameters related to the excluded biomarker are fixed according to Data Set S3. We argue that using the model in this way should be valid for data from drug-susceptible TB patients. However, for drug-resistant TB, the bacterial killing may be slower and initial bacterial load as well as growth rate in liquid culture may deviate, and studies investigating this are required. One of the most encouraging aspects of this model is that the parameter for the difference in bacterial kill for 35 versus 10 mg/kg rifampin was identifiable when using data from one or the other biomarker or when using data from both.

The original study (20) which reported the underlying data had not gone through formal peer-review by the time of manuscript submission of the present work.

The developed pharmacometric model predicted a general trend of lower probability of TTP culture conversion at week 8 for higher bacterial loads than for lower bacterial loads. This conclusion could probably not have been drawn as easily directly from the observed data. In the observed data, the mean baseline TTP was 5.7 days for patients with culture negativity at week 8, and the mean baseline TTP was 6.0 for patients with culture positivity at week 8. For other time points (including weeks 1, 4, and 6), the mean TTP was also similar between patients with and without culture conversion at week 8. However, for week 2, the mean TTP was higher for patients with culture negativity at week 8 (15.6 days) than that of patients with culture positivity at week 8 (9.8 days).

In conclusion, our work reports a practical combined MBL-TTP model that relates the changing bacterial load for both markers. We also developed two submodels that can be used to analyze TTP and MBL separately. The combined MBL-TTP model can be used to predict TTP from MBL data and *vice versa* and could be used to reanalyze historical trials. We confirm and delineate the extent that MBL gives a higher proportion of positive samples than TTP due to a high proportion of contaminated TTP samples. The standalone MBL model can be used to analyze clinical trials where exposure-response of drugs and regimens quantified with only MBL is of interest.

MATERIALS AND METHODS

Patient data. The model was developed on joint MBL and TTP observations collected repeatedly over the first 12 weeks of treatment in TB patients from an underlying study whose design and original findings are reported in detail in the relevant reference (20). Briefly, the data set was comprised of data from three clinical sites in Malawi, Mozambique, and Tanzania, with a total sample size of 105 patients (20, 53, and 32 patients, respectively). For the current analysis, only patients with drug-susceptible TB were included. The Tanzania data were a subset of the multiarm multistage TB (MAMS-TB) trial that has been described in detail elsewhere (21). All patients received rifampin and isoniazid throughout the whole study. Rifampin was given as 10 mg/kg in 93 patients and 35 mg/kg in 12 of the patients from MAMS-TB (21). Isoniazid was given as a standard dosage (5 mg/kg). Ethambutol and pyrazinamide were given as a standard dosage (15 to 20 and 20 to 30 mg/kg, respectively) for the first 8 weeks. Sputum was collected at baseline and at weeks 2, 4, 8, and 12 at all three sites. Sputum sampling was done either by spot sampling where sputum was collected during the on-going visit or by early morning samples where sputum was collected overnight. Pooled spot and early morning sputum were used to determine MBL and TTP for Malawi and Mozambique. For Tanzania, MBL was quantified on spot and TTP on early morning sputum. The procedure for MBL quantification was identical between sites as described previously (7). Time-to-positivity was determined using MGIT 960 (Becton, Dickinson, Sparks, MD). The TTP was tested for contamination for the Mozambique and Tanzania sites but not for Malawi. For the current analysis, samples with MBL below 100 CFU/ml were considered negative (i.e., the lower limit of quantification [LLOQ], 100 CFU/ml) (5) and TTP above 42 days were considered negative.

Modeling strategy overview. The main goal with the model development was to develop a final model that described the relationship between MBL and TTP data. However, the model development was divided into first developing a MBL-only model, after which TTP data were included in the modeling to develop a final, joint MBL-TTP model.

For the continuous MBL biomarker directly reflecting bacterial load, we considered analyzing this biomarker using models able to describe declining bacterial density in sputum, such as a biexponential

function, as applied previously to MBL data (5). The model for bacterial load was termed the sputum submodel.

The TTP data were analyzed in a different fashion (8) considering it is an indirect measurement of bacterial load reflecting time-to-event data. For TTP, the experimental procedure is first to inoculate bacteria in sputum in a liquid culture where growth takes place. This was described in our approach by linking the sputum model which describes changes in bacterial load in sputum to a mycobacterial growth submodel. The growth in liquid culture leads to carbon dioxide production and as the carbon dioxide reaches a certain level, a positive signaling event is recorded. Thus, a high degree of growth is expected to yield a high probability of achieving a short TTP, and this was handled in our approach by linking the growth to the probability of a positive signaling event to occur using survival modeling by incorporating a hazard submodel. Finally, a novel feature of this work is the addition of another submodel to account for contaminated TTP samples, which was implemented as a probability component to describe differences in the probability of TTP contamination over time and between the different study sites.

Modeling of MBL data. The MBL data were described through a sputum submodel describing the total bacterial load in the patient's sputum. The sputum model that was used as a starting point included a single mycobacterial subpopulation with exponential kill where bacterial load in sputum (B_s) over time on treatment (t_t) was calculated according to

$$B_s(t_t) = B_{0,s} \times e^{-k \times t_t} \quad (1)$$

where the MBL prediction was set equal to the bacterial load [i.e., $MBL(t_t) = B_s(t_t)$]. The $B_{0,s}$ parameter describes the initial (pretreatment) bacterial load and k is a first-order kill rate exhibited by the combination treatment. In this way, the drug effect was modeled as an "on/off" treatment effect not accounting for drug concentrations, i.e., this concentration-independent approach ignores pharmacokinetics. As this work developed, we tested a model that included two mycobacterial subpopulations ($B_{1,s}$ and $B_{2,s}$, respectively) with first-order rate constants for bacterial killing (k_1 and k_2 , respectively) according to

$$B_s(t_t) = B_{1,0,s} \times e^{-k_1 \times t_t} + B_{2,0,s} \times e^{-k_2 \times t_t} \quad (2)$$

where $B_{1,0,s}$ and $B_{2,0,s}$ describe the initial bacterial load of B1 and B2, respectively. In addition to the two-subpopulation model, a three-subpopulation model was also tested. As a molecular measure, we assumed that MBL captured a total population; thus, the prediction of MBL was set to the sum of the different bacterial subpopulations in sputum. The MBL data that were below the LLOQ which was set to 100 CFU/ml in this work was handled using the M3 method within NONMEM which is a preferred way to account for missing data (22).

Modeling of MBL-TTP data. For modeling TTP, the sputum submodel established based on the MBL data was extended with additional submodels (a schematic representation of how the different submodels connect can be seen in Fig. 1). Thus, the sputum submodel had a central role within the model and acted as the main driver for time-varying changes in both biomarkers. The starting point for model development of TTP-related submodels was derived from a previous TTP model (8). A mycobacterial growth component described bacterial growth in the liquid culture. The starting point for bacterial growth (the inoculum) for each liquid culture sample was the predicted bacterial load at the corresponding time point from the sputum submodel according to

$$B_s(t_t = t_{\text{sample}}) \rightarrow B_c(t_c = 0) \quad (3)$$

where t_{sample} is the time point of sampling (relative to start of treatment), B_c is the bacterial density in liquid culture, and t_c is time since liquid culture inoculation. In general for equations, t_t (time since start of treatment) signifies processes in the patient (e.g., bacterial killing), whereas t_c (time since MGIT inoculation) mainly concerns processes within the liquid culture. The existence of more than one mycobacterial subpopulation that we explored for the sputum model (e.g., in equation 2) was considered for the mycobacterial growth model also in which the starting point for bacterial growth was described by equations 4 and 5.

$$B_{1,s}(t_t = t_{\text{sample}}) \rightarrow B_{1,c}(t_c = 0) \quad (4)$$

$$B_{2,s}(t_t = t_{\text{sample}}) \rightarrow B_{2,c}(t_c = 0) \quad (5)$$

Upon exploring the existence of more than one subpopulation in the liquid culture, potential qualitative differences between subpopulations were tested, including different growth rates for the subpopulations and a transfer between subpopulations. Models were also tested including the existence of a nongrowing subpopulation (alongside a growing population) to explore if this could explain an expected time-varying change in the MBL versus TTP relationship (similar hypothesis exists for the CFU versus TTP relationship) (23). Exponential, logistic, and Gompertz growth functions were tested.

The mycobacterial growth model was coupled to a hazard model to translate growth in the MGIT liquid culture to a probability of a positive TTP signal.

Bacterial population density inside the liquid culture was the assumed contributor to the probability of a positive TTP signal. Bacterial population was an assumed proxy for carbon dioxide production, the known driver for a positive TTP signal (in this bacteria were assumed to be growing and this carbon dioxide producing). Note that no formal distinction was made between bacterial growth and carbon dioxide production, which means that the bacterial growth represents a combination of carbon dioxide production and bacterial growth. A scaling parameter controlled how much each bacterium inside the liquid culture contributed to the probability of a positive signal, as seen in equation 6 below

$$h(t_c) = B_c(t_c) \times \text{scale} \quad (6)$$

where h is the hazard and described the instantaneous probability for a positive signaling event and Scale is a scaling parameter controlling the contribution of each bacterium to the hazard. Next, the integral of the hazard over time (H) was calculated using equation 7:

$$H(t_c) = \int_0^{t_c} h(t_c) dt \quad (7)$$

The survival (S , the probability over time to remain free of a positive signaling event) was calculated by equation 8.

$$S(t_c) = e^{-H(t_c)} \quad (8)$$

For mycobacterial growth models including more than one mycobacterial subpopulation, we tested for differences in the degree of contribution to the probability of a positive TTP signal for each subpopulation.

Developing our work further, a component for the probability of contaminated TTP samples was developed. Tested models included constant (equation 9) and linearly increasing probabilities (equation 10) of contamination over time on treatment:

$$p_{\text{contaminated,TTP}} = p_{\text{con,base}} \quad (9)$$

$$p_{\text{contaminated,TTP}} = p_{\text{con,base}} + k_p \times t_t \quad (10)$$

where $p_{\text{contaminated,TTP}}$ is the probability of a contaminated TTP sample, $p_{\text{con,base}}$ is the baseline probability of a contaminated TTP sample, and k_p is a linear time-varying increase of probability of a contaminated TTP sample. Since the sputum sampling and testing for contamination differed between the sites, models were tested where separate contamination-related parameters were estimated for each site.

At the beginning of model development, negative TTP samples were handled within the time-to-event approach using right-censoring (the standard procedure for survival modeling). This was compared with a model where negative samples were handled by treating negative TTP samples as a different type of data observation in a separate submodel (10). The probability of a negative TTP sample was described by an E_{max} relationship between bacterial load in sputum and the probability of a negative TTP ($p_{\text{negative,TTP}}$) according to equation 11 (exemplified for a two subpopulation sputum model):

$$p_{\text{negative,TTP}} = 1 - \frac{p_{\text{max}} \times [B_{1s}(t_t) + B_{2s}(t_t)]^\gamma}{B_{50}^\gamma + [B_{1s}(t_t) + B_{2s}(t_t)]^\gamma} \quad (11)$$

where p_{max} is the maximal probability of a positive TTP sample, B_{50} is the bacterial load of subpopulation 1 and subpopulation 2 in sputum at which the probability of a positive TTP value is half maximal and γ is a gamma factor for the shape of the nonlinear relationship.

Covariate model. Rifampin dose groups of 35 versus 10 mg/kg were tested as a covariate on the bacterial kill rate in the sputum submodel as well as HIV status on baseline bacterial load. Another potential covariate to evaluate would be to test if pooled versus early morning sputum samples gave different baseline bacterial load (pooled samples are known to have shorter TTP), but this was not tested in this analysis. The reasons were that a graphical exploration of the data revealed no apparent differences between baseline TTP for pooled versus early morning samples and that all samples from each site had the same sampling. This would, in turn, have made it difficult to separate this effect between sampling method and site or region.

Another relevant covariate would have been lung cavitation on baseline bacterial load, but this information was unavailable in the current data set.

Utility of the model. The intended real-life use of the model was evaluated by reestimating the final combined MBL-TTP model by only using the MBL or TTP data, respectively, to explore if the final combined model can be applied to predict TTP from MBL data and *vice versa*, in trials where only MBL or TTP are collected. Note that this was an actual reestimation (i.e., not MAXEVAL of 0), but parameters strongly associated with the biomarker left out of the estimation were fixed to that of the combined MBL-TTP model. Furthermore, the model reestimated with TTP-only data can potentially be used as a standalone TTP model to analyze future TTP-only data sets (but note that this work does not include validation for prospective use *per se*). In this situation, the MBL submodel parameters were fixed to the estimates from the combined model when estimating only TTP data and *vice versa*. The evaluation was based on graphical diagnostic plots, plausibility of parameter estimates, and uncertainty in parameter estimates.

Data analysis and model evaluations. The data were analyzed in NONMEM 7.4 with the importance sampling (IMP) estimation method. The Laplacian estimation method did not give stable estimation for analyzing MBL and TTP simultaneously. Detailed estimation settings are listed in Data set S1 in the supplemental material. Data handling and plotting were done in R 3.5.1 using Xpose 4.6.1 (<http://xpose.sourceforge.net/>) to make diagnostic plots assisted by PsN 4.8.0 (<https://uopharmacometrics.github.io/PsN/>). Models were compared based on difference in objective function value (dOFV) using the likelihood ratio test at the 1% significance level but also based on uncertainty in model parameters.

Models were assessed graphically using visual predictive checks (VPCs). For MBL, conventional VPCs were generated which compared percentiles of observed and simulated data within the same plot. If the observed and simulated data agreed, it provided evidence that the model provided a good description of the observed data.

For TTP (time-to-event data), Kaplan-Meier VPCs (e.g., see reference 24) were produced, which compared observed and simulated Kaplan-Meier curves for TTP at each week. Finally, VPCs were performed for TTP versus MBL to assess if the model could mimic the observed pattern (relationship) between the biomarkers.

SUPPLEMENTAL MATERIAL

Supplemental material for this article may be found at <https://doi.org/10.1128/AAC.00652-19>.

SUPPLEMENTAL FILE 1, PDF file, 0.9 MB.

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