Pre-treatment cerebrospinal fluid bacterial load correlates with inflammatory response and predicts neurological events during tuberculous meningitis treatment

Nguyen TT Thuong[1], Dao N Vinh[1], Hoang T Hai[1], Do DA Thu[1], Le TH Nhat[1], Dorothee Heemskerk[1,2], Nguyen D Bang[3], Maxine Caws[1,4], Nguyen TH Mai[1,5], Guy E Thwaites[1,2]

1. Oxford University Clinical Research Unit, Ho Chi Minh City, Viet Nam

2. Nuffield Department of Medicine, University of Oxford, United Kingdom

3. Pham Ngoc Thach Hospital, Ho Chi Minh City, Viet Nam

4. Liverpool School of Tropical Medicine, Liverpool, United Kingdom

5. Hospital for Tropical Diseases, Ho Chi Minh City, Viet Nam

Running head: Bacterial load and TB meningitis outcome

Summary: In TB meningitis, higher pre-treatment CSF bacterial load increased disease severity and host inflammation. Bacterial load can be used to predict new neurological events but not death. Reducing neurological complications and deaths may need reassessment due to their divergent pathogenesis.

Footnote Page

Corresponding author: Dr Nguyen Thuy Thuong Thuong, Oxford University Clinical Research Unit, Hospital for Tropical Diseases, 764 Vo Van Kiet, W.1, Dist.5, Ho Chi Minh City, Vietnam; Tel: +84 8 924 1761; Fax: +84 8 923 8904; Email: thuongntt@oucru.org

Alternate corresponding author: Prof Guy Thwaites, Oxford University Clinical Research Unit, Hospital for Tropical Diseases, 764 Vo Van Kiet, W.1, Dist.5, Ho Chi Minh City, Vietnam; Email: gthwaites@oucru.org

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Abstract

Background: *Mycobacterium tuberculosis* (*Mtb*) bacillary load in the brain of those with tuberculous meningitis (TBM) may reflect the host ability to control the pathogen and determine disease severity and treatment outcomes.

Methods: We measured pre-treatment cerebrospinal fluid (CSF) *Mtb* bacterial load by GeneXpert in 692 adults with TBM. We sought to understand the relationship between CSF bacterial load and inflammation, and their respective impact on disease severity and treatment outcomes.

Results: Ten-fold higher *Mtb* load was associated with increased disease severity (Odds Ratio=1.59, p=0.001 for grade 1 versus grade 3), and increased CSF neutrophils (r=0.364, p<0.0001) and cytokine concentrations (r=0.438, p<0.0001). High *Mtb* load predicted new neurological events after starting treatment (Multinomial logistic regression, p=0.005), but not death. Death was previously associated with attenuated inflammatory response at the start of treatment, with reduced cytokine concentrations compared to survivors. In contrast, patients with high pre-treatment CSF bacterial loads, cytokines, and neutrophils were more likely to subsequently suffer neurological events.

Conclusions: Pre-treatment GeneXpert-derived *Mtb* load may be a useful predictor of neurological complications occurring during TBM treatment. Therapeutic strategies aimed at reducing neurological complications and deaths from TBM may need reassessment, given the evidence for their divergent pathogenesis.

Key words: bacterial load, tuberculous meningitis, inflammatory response, cytokines, neurological events

Introduction

Tuberculous meningitis (TBM) is the most severe form of tuberculosis (TB). It is caused by dissemination of *Mycobacterium tuberculosis (Mtb)* to the brain, resulting in meningo-encephalitis with necrotising, granulomatous inflammation predominantly affecting the basal meninges. Inflammation can lead to life-threatening complications of hydrocephalus, infarcts and tuberculomas (1, 2). Death or neurological disability still occur in half of all cases.

The pathogenesis of TBM is not well understood. Much of the pathology is thought to arise from the immune response to replicating bacteria, but the nature of that response and its relationship to bacterial numbers is presently poorly defined (3). Recent studies in large, well-characterised cohorts of HIV-uninfected Vietnamese adults with TBM demonstrated that elevated cerebrospinal fluid (CSF) cytokine concentrations in 8/10 tested cytokines were associated with more severe disease (4). Death, however, was associated with an attenuated inflammatory response, with lower CSF cytokine concentrations and leukocyte counts (4), and with higher CSF neutrophil counts (5). HIV infection, and polymorphism rs17525495 in the gene encoding leukotriene A4 hydrolase (*LTA4H*), influenced both pretreatment CSF inflammatory phenotype and survival from TBM in Vietnamese individuals (4), but the polymorphism was not associated with survival in Indonesians (5).

Neutrophils appear to play an important role in TBM pathogenesis (5). Higher CSF neutrophil numbers have been associated with an increased likelihood of culturing *Mtb* from the CSF (6, 7) and cerebral immune reconstitution inflammatory syndrome (IRIS) in those co-infected with HIV (8, 9).

One of the fundamental questions concerning TBM pathophysiology is how *Mtb* bacterial load relates to intracerebral inflammation and outcome. Very low bacterial numbers in CSF, and inadequate laboratory methods, have to date made this question largely refractory to

investigation. CSF culture positivity may indicate higher bacterial loads, and was associated with mortality in HIV-uninfected TBM patients (5). More recent molecular assays, such as GeneXpert, quantify the presence of bacterial nucleic acid in CSF and are now widely used for TBM diagnosis. Although GeneXpert's sensitivity for TBM diagnosis is around 60% compared to clinical diagnosis (12), when positive it categorizes CSF bacterial load as very low, low, medium, or high, thus offering a new way to assess CSF *Mtb* load in clinical practice. The aim of our study was to use GeneXpert to define CSF bacterial load in a large cohort of well-characterised Vietnamese adults with TBM; and to investigate the relationship between bacterial load and CSF cytokine concentrations, leucocyte numbers and types, and the occurrence of new neurological events and death after the start of anti-tuberculosis treatment.

Methods

Participants

Adults (>17 years) with TBM were enrolled into a randomized controlled trial of intensified anti-tuberculosis chemotherapy between April 2011 and June 2014 (10). Of the 817 trial participants, 692 had CSF GeneXpert data and were included in the current study. The other 125 participants had missing data due to insufficient volume of CSF samples or GeneXpert test errors.

Written informed consent was obtained from each participant or from an accompanying relative if the participant could not provide consent. Protocols were approved by the Oxford Tropical Research Ethics Committee in the United Kingdom, the institutional review boards of the Hospital for Tropical Diseases and Pham Ngoc Thach Hospital for Tuberculosis and Lung Disease, and the Ethical Committee of the Ministry of Health in Vietnam.

Treatment

Participants were randomly allocated to treatment with (a) standard anti-tuberculosis regimen for 3 months, followed by rifampicin and isoniazid at the same doses for a further 6 months, or (b) an intensified regimen that consisted of the standard regimen with an additional higher dose of rifampicin (15 mg/kg/day) and levofloxacin (20 mg/kg/day) for the first 8 weeks of treatment. All participants also received adjunctive dexamethasone for the first 6–8 weeks of treatment (10).

Clinical and CSF characteristics

We extracted data on participant age, weight, duration of illness (days of symptoms), Glasgow coma score (GCS), British Medical Research Council (BMRC) grading for TBM, TB treatment and HIV status on the date of TBM diagnosis. All participants were followed for 9 months, with careful characterization of their clinical response to treatment. New neurological events were defined as the new occurrence of any of the following: cerebellar symptoms, mono-, hemi-, para, or tetraplegia, seizures, cranial nerve palsy, or a fall in GCS by ≥ 2 points for ≥ 2 days from the highest previously recorded score (10). These events encompass paradoxical treatment reactions, which are thought to be caused by an excessive inflammatory response to dead or dying bacteria (11) and death of brain tissue.

Laboratory data were collected from samples taken on the date of enrollment, including blood and CSF cell counts, and cytokine concentrations. A panel of 10 cytokines important in inflammation in TB, comprising six pro-inflammatory cytokines (interleukin1β (IL-1β), IL-2, IL-6, IL-12p70, interferon γ (IFN-γ), and tumor necrosis factor α (TNF-α)) and four anti-inflammatory cytokines (IL-4, IL-5, IL-6 and IL-13), was measured in stored CSF specimens using Luminex multiplex bead-based assay (4). *LTA4H* rs17525495 polymorphism was genotyped by TaqMan genotyping assay (3).

GeneXpert MTB/RIF, which detects *Mtb* complex and rifampicin resistance, was performed on CSF before the start of anti-TB treatment (12). Briefly, 3-5 ml of CSF was centrifuged for 20 minutes at 3000g; 200 µl(about one-third of the deposit) was resuspended in phosphate-buffered saline and mixed with 1.5 ml of sample reagent, vortexed for 30 seconds, incubated for 15 minutes at room temperature then transferred to a GeneXpert cartridge for measurement. The GeneXpert Dx software (version 4.0; Cepheid) reported semiquantitative mycobacterial load results as cycle threshold (Ct) values, representing the number of PCR cycles required for the signal to reach a detection threshold. *Mtb* loads were classified as high (Ct <16), medium (Ct 16-22), low (Ct 22-28), and very low (Ct >28). *Mycobacterium bovis* (BCG, NCTC 5692) was used to generate a standard curve of GeneXpert Ct values versus bacterial number by the Miles and Misra method (13). For each sample, the average Ct value of five probes (excluding any delayed values due to rifampicin resistance) was used to estimate bacterial load and analyse further.

Statistical Analysis

Data were analysed using statistical package R v3.0.1 (14), MATLAB and Statistics Toolbox Release 2013a (The MathWorks Inc., Natick, Massachusetts, USA), and Graphpad Prism version 6 (Graphpad Software Inc., San Diego, California, USA). In statistical analyses, to preserve the statistical power we used continuous comparisons of the whole dataset, with GeneXpert negative assigned a Ct value of 40; meanwhile most of the graphical representations use bacterial load in categories for clarity.

Baseline concentrations of the ten CSF cytokines were analysed using principal component analysis (PCA), a method of transforming complex correlated datasets into a new coordinator system (component space) in which two or three first principle components can cover up to 70% - 80% variance of the whole dataset. By this means we visualized our multi-variate dataset and

avoided multiple comparisons (15, 16). Associations between *Mtb* load and cytokine principal components (PCs), blood or CSF cell counts were examined using Spearman rank correlation coefficient, in which log-transformed cell counts were used to analyse correlations. Comparisons of bacterial loads by GCS or disease severity, CSF neutrophils and cytokines were performed by a linear trend test implemented using robust linear regression. We classified outcomes into three groups: (1) 'survival' means participants survived without new neurological events, (2) 'new neurological events' means they survived with new neurological events occurring during treatment and (3) 'death' means they died during treatment. Comparisons of bacterial load between survival and new neurological events or death utilized Mann-Whitney tests. The outcome was modeled using multinomial logistic regression depending on *Mtb* bacterial load and the detection limit indicator. The model was later adjusted for predefined risk factors including age, weight, GCS, CSF leukocyte numbers, anti-tuberculosis regimen, anti-retroviral therapy, HIV infection, and *LTA4H* genotype. Separate analysis was also performed for each HIV status. We used the Hochberg method to correct p-values for multiple testing.

Results

Characteristics of the participants

The baseline clinical characteristics of the 692 TBM adults with CSF *Mtb* load assessed by GeneXpert are described in Table 1. 293 (42.3%) were GeneXpert positive and these were significantly more likely than the GeneXpert negative participants to be HIV-infected and to have more severe disease. The majority of participants (568, 82.1%) had BMRC grade 1 or 2 disease at enrollment. The detection of *Mtb* in CSF by microscopy and MGIT culture were 257 (37.6%) and 281 (41.2%). In the 228 patients positive in both GeneXpert and MGIT culture,

correlation between the bacillary load and the days to culture positivity was 0.417, p<0.0001. Participants who were GeneXpert positive had higher numbers of leucocytes, percentage of neutrophils, and levels of protein and lactate in CSF than those who were GeneXpert negative. Clinical characteristics of HIV-uninfected and -infected patients and their blood and CSF parameters are shown in Table S1.

Positive detection of *Mtb* by GeneXpert in HIV-infected participants (165/288, 57.3%) was higher than in HIV-uninfected participants (128/404, 31.7%) (p<0.0001), although *Mtb* loads in these two groups were not significantly different (p=0.087). Pre-treatment GeneXpert CSF bacterial loads varied from very low to medium; a high load was not recorded. In the 404 HIV-uninfected participants, 276 (68.3%) had a negative test, 63 had very low bacterial loads (15.6%), 58 low loads (14.4%), and 7 medium loads (1.7%). In the 288 HIV-infected participants, 123 (42.7%) had a negative test, 72 had very low bacterial loads (25.0%), 74 low loads (25.7%), and 19 medium loads (6.6%) (Figure 1). Conversions from Ct value to bacterial number, and ranges of bacterial number by levels of bacterial load, can be found in Figure S1.

Association between pre-treatment *Mtb* load and disease severity

The associations between *Mtb* load and GCS or BMRC grade were examined before treatment in 692 TBM adults. *Mtb* loads showed positive correlations with GCS (Spearman correlation: HIV-uninfected, r= 0.194, p < 0.0001; HIV-infected, r= 0.238, p< 0.0001) , i.e. higher bacterial loads in those with more severe coma, and increased *Mtb* load was associated with lower GCS (linear trend test: HIV-uninfected, p= 0.0004; HIV-infected, p< 0.0001; Figure 2A).

In addition, increased *Mtb* load was also associated with more severe disease assessed by BMRC grade (linear trend : HIV-uninfected, p= 0.008; HIV-infected, p= 0.001). Bacterial load (per 10-fold increase) affected disease severity in all participants [grade 1 vs. grade 2, odds ratio (OR) 0.71, 95% confidence interval (CI) 0.91-1.50, p= 0.226; grade 1 vs. grade 3, OR 1.59, 95% CI 1.19-2.15, p= 0.001]; in HIV-uninfected participants (grade 1 vs. grade 2, OR 1.56, 95% CI 1.02-2.47, p= 0.039; grade 1 vs. grade 3, OR 1.84, 95% CI 1.12-3.21, p= 0.015), and in HIV-infected participants (grade 1 vs. grade 2, OR 1.0, 95% CI 0.7-1.4, p= 0.878; grade 1 vs. grade 3, OR 1.5, 95% CI 1.1-2.2, p= 0.013). The duration of illness before treatment was not associated with BMRC grade (p= 0.977) or *Mtb* load (p= 0.651).

Correlations between pre-treatment CSF *Mtb* load and inflammation

To investigate whether *Mtb* load before treatment was related to inflammatory responses we first explored the relationship between *Mtb* load and blood and CSF cell counts. Bacterial load by Ct values showed negative correlations with neutrophil numbers in blood and CSF in both HIV-infected and uninfected participants (Table 2, Figure 2B). These correlations were stronger in CSF ($r = -0.395$, $p < 0.0001$, Figure S3) than in blood (Table 2), suggesting that neutrophil recruitment correlated more strongly with bacterial replication at the infected site.

PCA was applied to ten baseline CSF cytokines to identify correlated measurements that account for the variance of the data set. The first component, PC1, covered 71.4% variance of the cytokine profile, PC2 covered 6.4%, PC3 4.9%, PC4 4.4% and other components covered less (Figure S2). In other words, the majority of the variance was reflected by just the two first principal components (PC1 and PC2). Because all the coefficients were located in quadrant I and quadrant IV, a positive value of PC1 implies high concentration across all cytokines. In other words, these cytokines were in strong co-correlation with each other. Meanwhile the PC2

explains the difference in cytokine concentrations of two groups: IL-13, IFN-γ, IL-10, IL-5 versus IL-2, IL-6, IL-1β. We used the first two components to represent the cytokine profile to assess whether bacterial load was related to cytokine concentrations. *Mtb* load by Ct values showed a negative relationship with PC1 overall ($r = -0.493$, $p < 0.0001$) and in participants stratified by HIV infection ($p < 0.0001$, $r = -0.448$ and -0.487 for HIV-uninfected and infected respectively, Table 2). *Mtb* loads were not correlated with PC2 in participants stratified by HIV. These results suggest that participants with high *Mtb* load tended to have high concentrations of measured cytokines. This can be seen particularly in the strong relationship between *Mtb* load and the key pro-inflammatory cytokines IL-1β and TNF-α (Table 2, Figure 2 C, D, Figure S3). Interestingly, CSF neutrophil count was positively correlated with PC1 regardless of HIV infection (all TBM, $r= 0.381$, $p< 0.0001$; HIV-uninfected, $r= 0.385$, $p< 0.0001$; HIV-infected, $r= 0.317$, $p= 0.0003$).

Association between *LTA4H* genotype and *Mtb* load

Previously, we showed that in HIV-uninfected adults with TBM, *LTA4H* genotype was associated with CSF IL-1β, IL-2, and IL-6 concentrations, with low concentrations in genotype CC, intermediate concentrations in CT, and high concentrations in TT (4). Laarhoven *et al.* reported that TT genotype was associated with decreased CSF culture positivity (5). We analysed the relationship of *LTA4H* genotype and bacterial load, and found *Mtb* load in participants with TT-genotype was slightly lower than that in CC ($p = 0.051$) or CT ($p = 0.015$), however the linear trend test showed *LTA4H* genotype was not associated with *Mtb* load overall ($p = 0.302$, Figure S4).

Relationship between CSF *Mtb* load, inflammation, and new neurological events

Study participants were treated with intensive (n=347) or standard anti-TB treatment (n=345). As previously reported, the intensive regimen was not associated with any improvement in any measure of treatment response or outcomes, including survival (10). During 9 months of anti-tuberculosis treatment, new neurological events developed in 103 participants (14.9%), the median time to new events after the start of treatment was 9 days (IQR 3-43) (Figure S5A). New neurological events were significantly associated with higher pre-treatment *Mtb* loads in the GeneXpert positive dataset (p = 0.004 in HIV-uninfected, p = 0.022 in HIV-infected, Figure 3A). In the whole dataset, elevated *Mtb* load increased risk of new neurological events (OR= 1.68, 95% CI 1.26-2.25 per 10-fold bacterial increase, p= 0.0004, Table 3). In both HIV-uninfected and infected participants, these events were also associated with high baseline CSF neutrophil counts, high baseline PC1 score and high baseline TNF-α concentrations (Figure 3B, C, D).

The pathogenesis of neurological events may differ according to their timing during treatment. Therefore, we categorized participants by the median time to new neurological events < 9 days or ≥ 9 days, and compared bacterial load between these two groups. Bacterial loads were not significantly different between early and late neurological events (p = 0.196 in HIV-uninfected, p = 0.061 in HIV-infected, Figure S6A).

Predictors of new neurological events

The results (Table 3) indicated that increased *Mtb* load was independently associated with new neurological events in all participants (OR 1.56, 95% CI 1.14–2.11, p= 0.005 and adjusted p= 0.045; per 10-fold load increase). *Mtb* load was significantly associated with new neurological events in HIV-uninfected participants (OR 1.97, 95% CI 1.18–3.28, p= 0.009) and with a trend in HIV-infected participants (OR 1.43, 95% CI 0.95–2.15, p= 0.082). Results stratified by HIV infection lost statistical significance after adjustment for multiple testing. GCS was also strongly

associated with new neurological events (OR 0.77, 95% CI 0.71–0.84, p < 0.0001; per point increase). These findings highlight that pretreatment CSF *Mtb* load can be used as a predictor, together with GCS, of new neurological events occurring during TBM treatment.

Relationship between CSF *Mtb* load, inflammation, and death

Death occurred in 192 (27.7%) participants, a median of 39 days (IQR 9-103) after the start of treatment (Figure S5B). Bacterial load was not significantly associated with death, with or without neurological events, in either HIV-uninfected or infected participants in the GeneXpert positive dataset (Figure 3A). Although CSF neutrophil counts were similar between those who died and survived (Figure 3B), measured cytokine concentrations were significantly lower in those who died, with or without neurological events, in HIV-uninfected participants (Figure 3C and D) in the whole dataset. To examine whether early death was associated with bacterial load, we categorized participants by the median time to death < 39 days or ≥ 39 days. Bacterial loads were not significantly different between those dying early or late (HIV-uninfected, p = 0.731; HIV-infected, p= 0.099; Figure S6B).

Taken together, our data showed that high *Mtb* load was associated with more severe disease at baseline and strongly predicted new neurological events after the start of treatment, but did not influence 9-month mortality (Table 3).

Discussion

TBM is one of the most difficult forms of TB to treat, with outcomes dependent upon killing the bacteria with anti-tuberculosis drugs and controlling intracerebral inflammation. However, the relationships between bacterial numbers, inflammation, and treatment response are poorly defined, primarily because of the difficulties assessing *Mtb* load at the site of infection. We overcame some of these difficulties by quantifying bacterial numbers in CSF by GeneXpert in a

large cohort of well-characterized patients recruited to a randomized controlled trial of intensive anti-tuberculosis therapy (10). We found that pretreatment CSF *Mtb* load was associated with increased CSF neutrophil numbers, enhanced inflammation and disease severity, and predicted the likelihood of new neurological events after the start of anti-TB treatment.

CSF bacterial load showed significant correlations with neutrophil numbers in blood and CSF in both HIV-infected and uninfected participants. Previously, CSF neutrophil numbers have been associated with positive CSF *Mtb* cultures (6, 17) and studies in HIV-coinfected individuals have linked the presence of *Mtb* in the CSF with a neutrophil-mediated inflammatory response and an increased risk of intracerebral IRIS (9). Neutrophils may be protective in early *Mtb* infection (18), although animal models suggest they may exacerbate pathology in the later stages of disease (19, 20). Clinical studies in active TB have suggested neutrophils may influence disease progression and outcome: higher blood neutrophil counts before treatment have been associated with increased risk of mortality in TB patients (of which 49.4% were pulmonary TB) (21) and slower sputum conversion to negative during therapy in pulmonary TB (22). In transcriptional profiling of blood, the signature of active TB patients was characterised by a neutrophil-driven interferon inducible gene expression (23). Taken together, these findings all suggest that neutrophils play an important role in TB pathogenesis.

Almost half the participants suffered serious clinical complications during treatment (new neurological events in 14.9%; death in 27.7%). Surprisingly, we found that CSF *Mtb* load influenced the likelihood of new neurological events, but not mortality, suggesting different underlying mechanisms. In support of this assertion, current and previous data suggest new neurological events are associated with high concentration of CSF cytokines before the start of treatment, whilst death is associated with attenuated inflammatory responses (4).

One of the limitations of our study was that we were unable to define the likely cause of the new neurological events by brain imaging. However, given the nature and timing of the events, and their association with increased inflammation, it is reasonable to assume that many could be defined as 'paradoxical' treatment reactions (11). Therefore, taken together with the recent finding by Marais et al (9), our data provides further evidence for the importance of bacterial load and neutrophils in treatment-associated inflammatory complications in HIV-infected and uninfected adults with TBM. Our findings support the hypothesis that the pathogenesis of IRIS and paradoxical reactions are similar, resulting from excessive neutrophil-mediated inflammation and driven by *Mtb* load. Drugs with the potential to target neutrophils (e.g. roflumilast, ibuprofen, doxycycline (11)) may be more effective than corticosteroids in the treatment of these common complications.

An additional limitation of our study was that we could not follow the decline of bacterial loads after the start of treatment. Even before the start of anti-tuberculosis treatment CSF bacillary loads are very low. In our participants, pre-treatment bacillary loads in CSF were at least a hundred-fold lower than loads reported in sputum ofpulmonary TB patients(24). Furthermore, repeated CSF sampling early in treatment is rarely clinically justifiable, which further restricts the information available. We routinely sample CSF after 30 and 60 days of therapy, by which time bacterial loads are almost always below the detection threshold.

In summary, our data shed new light on the pathogenesis of TBM and suggest divergent mechanisms that lead to death and neurological events occurring after the start of treatment. Death from TBM is associated with an attenuated inflammatory response at the start of treatment, with reduced CSF leucocytes and cytokine concentrations compared to survivors. In contrast, patients with high pre-treatment CSF bacterial loads, cytokines, and neutrophils are

more likely to suffer new neurological events or ‘paradoxical’ inflammatory complications. These findings may have important implications for the selection of future host directed therapies.

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Figure legends

Figure 1. Bacterial load in TBM participants uninfected or infected with HIV before the start of anti-tuberculosis treatment

Percentages of *Mtb* levels, including not-detected (ND), very low, low and medium, in 404 HIV-uninfected and 288 HIV-infected patients.

Figure 2. Associations between bacterial load and disease severity, CSF neutrophil counts, and CSF cytokine concentrations in TBM participants before the start of anti-tuberculosis treatment

GCS, Glasgow coma score; ND, not-detected

Data are for *Mtb* levels at baseline in 404 HIV-uninfected patients (276 ND, 63 very low, 58 low and 7 medium) and in 288 HIV-infected participants (123 ND, 72 very low, 74 low and 19 medium). Associations between baseline *Mtb* levels and (A) GCS (B) CSF neutrophil counts, (C) CSF IL-1β and (D) CSF TNF-α; p-values were derived from linear trend test. Long bars in plots represent median and short bars were below and/or above inter-quantile range values.

Figure 3. Associations between pre-treatment CSF bacterial load, inflammation, and new neurological events or death

(A) Comparison of the number of bacteria in CSF using the Ct value which is inversely proportional to bacterial load (i.e. the lower the value, the higher the load) in survivors [S], those with new neurological events [N] and those who died [D] in HIV-infected and HIV-uninfected participants. Numbers of cases were presented in brackets. (B) Comparison of numbers of neutrophils in CSF at baseline in HIV-infected and HIV-uninfected participants who survived,

suffered new neurological events, or died. (C, D) Comparison of CSF cytokine concentrations PC1 or TNF-α in HIV-uninfected in HIV-infected participants who survived, suffered new neurological events, or died. $p^{\#}$, comparison of S vs. D; $p^{\wedge}$, comparison of N vs. D. Bars in plots represent median and inter-quantile range values. Statistical comparisons between survival versus new neurological events or death were made using Mann-Whitney tests. CSF neutrophil and cytokine data were available for 657 and 554 patients respectively.

Table 1. Baseline clinical characteristics of all 692 TBM participants by CSF GeneXpert

Characteristic	All n	All Summary statistic	GeneXpert negative n	GeneXpert negative Summary statistic	GeneXpert positive n	GeneXpert positive Summary statistic	p values
Age (years) – median (IQR)	692	35 (29, 46)	399	36 (29, 50)	293	35 (29, 42)	0.008
Male sex – no. (%)	692	475 (68.6)	399	261 (65.4)	293	214 (73.0)	0.038
Weight (kg) – median (IQR)	692	48 (44, 54.5)	399	49 (45, 55)	293	48 (43, 54)	0.144
Duration of illness (days) – median (IQR)	692	15 (10, 30)	399	15 (10, 30)	293	15 (10, 30)	0.763
Glasgow coma score – median (IQR)	692	15 (12, 15)	399	15 (13, 15)	293	14 (11, 15)	<0.0001
HIV infection - *Positive* – no. (%)	692	288 (41.6)	399	123 (30.8)	293	165 (56.3)	<0.0001
Treatment arm - *Standard arm* – no. (%)	692	345 (49.9)	399	199 (49.9)	293	146 (49.8)	1
[#]BMRC Grade – no. (%)	692		399		293		0.001
-Grade I		266 (38.4)		162 (40.6)		104 (35.5)	
-Grade II		302 (43.6)		184 (46.1)		118 (40.3)	
-Grade III		124 (17.9)		53 (13.3)		71 (24.2)	
Other diagnostic tests							
-CSF-Smear - *Positive* – no. (%)	684	257 (37.6)	394	69 (17.5)	290	188 (64.8)	<0.0001
-CSF-MGIT culture - *Positive* – no. (%)	682	281 (41.2)	389	53 (13.6)	293	228 (77.8)	<0.0001
*Diagnostic category – no. (%)	692		399		293		<0.0001
-Definite TBM		397 (57.4)		104 (26.1)		293 (100)	
-Probable TBM		170 (24.6)		170 (42.6)		0 (0)	
-Possible TBM		125 (18.0)		125 (31.3)		0 (0)	
Cerebrospinal fluid - median (IQR)							
-Leucocytes total (x 10^3 cells/ml)	686	115 (35, 284)	395	76 (22, 198)	291	200 (64, 388)	<0.0001
-Neutrophils (%)	655	10 (0, 32)	371	3 (0, 15)	284	20 (5, 56)	<0.0001
-Lymphocytes (%)	65	90.0 (68,	37	97.5 (85,	28	79.5 (44, 95)	<0.00

	6	100)	2	100)	4		01
-Protein (g/l)	66		37		28		<0.00
	6	1.2 (0.6, 1.9)	9	1.0 (0.5, 1.7)	7	1.4 (0.9, 2.4)	01
-Glucose (mmol/l)	66		37		28		<0.00
	6	1.9 (1.3, 2.7)	9	2.2 (1.4, 3.0)	7	1.6 (1.0, 2.2)	01
-Lactate (mmol/l)	63		35		28		<0.00
	8	4.8 (3.5, 6.5)	7	4.1 (2.9, 5.6)	1	5.9 (4.5, 7.4)	01

All summary statistics are absolute counts (%) for categorical variables and median (inter-quartile range = IQR) for continuous data. n refers to the number of patients with non-missing data for the corresponding variable.
p values are descriptive only and based on χ^2 test for categorical data and Mann-Whitney test for continuous data.
[#]BMRC denotes modified British Medical Research Council criteria. Grade I indicates a GCS of 15 with no neurologic signs (baseline), grade II a score of 11 to 14 (or 15 with focal neurologic signs), and grade III a score of 10 or less.
*Diagnostic categories were assigned according to the consensus case definition.[1] Patients with an unlikely diagnosis of tuberculous meningitis had a score of <6. Confirmed other diagnosis was only made based on microbiological evidence (Marais et al.; The Lancet Infectious diseases. 2010; 10(11): 803-12)

Table 2. Relationship of *Mtb* load and white cell numbers and cytokines in blood and CSF from TBM patients by Spearman correlation

Patient group	Blood			Cerebrospinal fluid						
	Leucocytes	Lymphocytes	Neutrophils	Leucocytes	Lymphocytes	Neutrophils	IL-1β	TNF α	PC1	PC2
All TB, n	650	649	650	682	653	651	521	522	518	518
r	-0.027	**0.242**	-0.085	**-0.273**	**-0.11**	**-0.395**	**-0.523**	**-0.48**	**-0.493**	**0.185**
p value	0.486	< 0.0001	0.031	< 0.0001	0.005	< 0.0001	< 0.0001	< 0.0001	< 0.0001	< 0.0001
Adjusted p value	0.486	< 0.0001	0.062	< 0.0001	0.014	< 0.0001	< 0.0001	< 0.0001	< 0.0001	< 0.0001
HIV-uninfected, n	382	382	382	401	390	389	304	304	300	300
r	**-0.116**	**0.24**	**-0.182**	**-0.199**	-0.048	**-0.342**	**-0.456**	**0.413**	**-0.448**	0.127
p value	0.024	< 0.0001	< 0.0001	< 0.0001	0.343	< 0.0001	< 0.0001	< 0.0001	< 0.0001	0.028
Adjusted p value	0.056	< 0.0001	0.001	0.0003	0.343	< 0.0001	< 0.0001	< 0.0001	< 0.0001	0.056
HIV-infected, n	268	267	268	281	263	262	217	218	218	218
r	**-0.164**	0.129	**-0.204**	**-0.35**	**-0.206**	**-0.388**	**-0.499**	**0.515**	**-0.487**	0.114
p value	0.007	0.035	0.001	< 0.0001	0.001	< 0.0001	< 0.0001	< 0.0001	< 0.0001	0.092
Adjusted p value	0.021	0.070	0.003	< 0.0001	0.003	< 0.0001	< 0.0001	< 0.0001	< 0.0001	0.092

Analyses were performed by using GeneXpert Ct values and $\log_{10}$-transformed cell counts, IL-1β and TNF-α
r: Spearman's rho correlation coefficient, in bold if adjusted $p < 0.05$. p values were adjusted with the Hochberg method.

Table 3. Association between pre-treatment variables and new neurological events and death by multinomial logistic regression analysis

Variables	All patients (n = 692)		HIV-uninfected (n = 404)		HIV-infected (n = 288)	
	HR (95% CI)	**p value**	**HR (95% CI)**	**p value**	**HR (95% CI)**	**p value**
New neurological events (*Mtb* load effect)						
Mtb load (per 10-fold increase)	1.68 (1.26-2.25)	0.0004	2.02 (1.26-3.23)	0.003	0.67 (0.16-0.98)	0.038
Mtb below LOD	3.12 (0.96-10.1)	0.057	6.83 (1.10-42.4)	0.039	1.32 (0.25-7.03)	0.745
New neurological events (adjusted effect) †						
Mtb load (per 10-fold increase) §	1.56 (1.14-2.11)	0.005	1.97 (1.18-3.28)	0.009	1.43 (0.95-2.15)	0.082
Mtb below LOD	2.84 (0.83-9.68)	0.096	7.60 (1.05-54.9)	0.044	1.55 (0.27-8.75)	0.617
Age (per 10-y increase)	1.07 (0.91-1.27)	0.416	1.09 (0.90-1.31)	0.367	1.16 (0.67-2.03)	0.593
Weight (per 10-kg increase)	1.14 (0.87-1.49)	0.325	1.04 (0.74-1.46)	0.831	1.33 (0.83-2.12)	0.228
Glasgow coma score (per point increase)	0.77 (0.71-0.84)	< 0.0001	0.77 (0.69-0.85)	< 0.0001	0.75 (0.63-0.89)	0.001
CSF leukocyte count (per 10-fold increase)	1.00 (0.99-1.00)	0.552	1.00 (0.99-1.00)	0.733	1.00 (0.99-1.01)	0.393
Intensified regimen	0.84 (0.53-1.33)	0.453	0.53 (0.29-0.96)	0.037	1.76 (0.80-3.90)	0.159
HIV infected	1.07 (0.64-1.80)	0.791	…		…	
Antiretroviral therapy at enrolment	…		…		1.83 (0.80-4.18)	0.149
LTA4H genotype: CC vs TT	1.08 (0.51-2.28)	0.830	1.36 (0.49-3.77)	0.547	0.65 (0.20-2.10)	0.473
LTA4H genotype: CT vs TT	0.96 (0.46-2.02)	0.915	0.84 (0.30-2.34)	0.741	0.9 (0.28-2.86)	0.856
Mortality (*Mtb* load effect)						
Mtb load (per 10-fold increase)	1.49 (1.15-1.92)	0.002	1.54 (0.96-2.48)	0.072	0.73 (0.54-0.99)	0.045
Mtb below LOD	3.06 (1.14-8.22)	0.026	3.82 (0.66-22.1)	0.134	3.22 (0.94-11.05)	0.063
Mortality (adjusted effect)						
Mtb load (per 10-fold increase) ¶	1.25 (0.94-1.68)	0.127	1.43 (0.82-2.50)	0.207	1.14 (0.79-1.64)	0.480
Mtb below LOD	2.76 (0.89-8.53)	0.077	2.72 (0.35-21.3)	0.341	2.45 (0.58-10.2)	0.220
Age (per 10-y increase)	1.52 (1.30-	<	1.68 (1.40-	<	0.91 (0.59-	0.675

	1.78)	0.0001	2.01)	0.0001	1.41)	
Weight (per 10-kg increase)	0.67 (0.51-0.88)	0.003	0.87 (0.59-1.27)	0.475	0.58 (0.39-0.88)	0.010
Glasgow coma score (per point increase)	0.69 (0.64-0.75)	<0.0001	0.71 (0.64-0.80)	<0.0001	0.65 (0.56-0.75)	<0.0001
CSF leukocyte count (per 10-fold increase)	1.00 (0.99-1.00)	0.270	0.98 (0.97-1.00)	0.094	1.00 (0.99-1.00)	0.933
Intensified regimen	0.91 (0.60-1.38)	0.667	0.77 (0.42-1.41)	0.405	1.15 (0.64-2.08)	0.637
HIV infected	5.50 (3.38-8.96)	<0.0001	…		…	
Antiretroviral therapy at enrolment	…		…		0.80 (0.43-1.50)	0.487
LTA4H genotype: CC vs TT	2.24 (1.06-4.76)	0.033	3.82 (1.02-14.3)	0.046	1.66 (0.64-4.35)	0.297
LTA4H genotype: CT vs TT	1.86 (0.87-3.96)	0.108	2.04 (0.54-7.65)	0.289	1.83 (0.69-4.87)	0.225

Abbreviation: HIV, Human Immunodeficiency Virus; CI, confidence interval; CSF, cerebrospinal fluid; HR, hazard ratio; LOD, limit of detection

Mtb load below LOD if GeneXpert was negative. *Mtb* load covariate and indicator of LOD were used in the model.

† Adjusted effect means adjusted for risk factors in the multivariate model.

§ p values adjusted by the Hochberg method for association of *Mtb* load and new neurological events: p = 0.045 (all patients), p = 0.075 (HIV-uninfected), p = 0.823 (HIV-infected).

¶ p values adjusted by the Hochberg method for association of *Mtb* load and death: p = 0.382 (all patients), p = 0.475 (HIV-uninfected), p = 0.933 (HIV-infected).