Evaluation of new biomarker-based approaches for improving the diagnosis of childhood tuberculous meningitis

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Literature review

Introduction

Tuberculous meningitis (TBM) is the most common type of bacterial meningitis in the Western Cape Province (WCP) of South Africa [1]. The tuberculosis (TB) incidence in South Africa has risen from 301 new cases/100,000 population in 1990 to 948 new cases/100,000 population in 2007 [2], and although the world-wide incidence of the disease has fallen by an average of 1.5% per year since 2000, the incidence of the disease in South Africa is still 834 per 100, 000 population as indicated by recent World Health Organization (WHO) reports [3]. The TB burden is worsened by the human immunodeficiency virus (HIV) pandemic. TBM represents the most severe manifestation of TB with high morbidity and mortality, despite adequate anti-TB therapy. This is mainly the result of delayed diagnosis and initiation of appropriate therapy [4]. The pathogenesis of TB is still poorly understood. Despite ongoing research, early, cost-effective diagnostic tools are lacking [5]. Demonstration of acid-fast bacilli and/or culture of *Mycobacterium tuberculosis* (*Mtb*) from cerebrospinal fluid (CSF) represents the gold standard for diagnosing TBM, but unfortunately the sensitivity of both these tests is low [6, 7]. The sensitivity of the recently developed GeneXpert

test (Cepheid Inc., USA) for TBM is ˜50-60%, but only improves to 72% when CSF is centrifuged as demonstrated in a recent study [8]. The diagnosis of TBM is therefore based on a combination of clinical, laboratory and radiological findings. However, a clinical diagnosis is often delayed, especially in the early stages of the disease when few neurological signs may be present. In addition, the CSF picture can be highly variable [9]. Although various case definitions for TBM have been reported in the literature, diagnostic criteria vary widely, so that no standardized case definition exists [6, 7, 9-11]. An international workshop on TBM was held during May 2009 in Cape Town, South Africa. This was attended by international experts on TBM, and a uniform case definition for TBM was proposed for future research.

Uniform research case definition for TBM [12]

It is proposed that patients suspected of having TBM be categorized into definite, probable, possible and no TBM. This is proposed to obtain standardization for research purposes, for adults and children, and was not primarily devised as a clinical tool. The clinical diagnosis of TBM (probable or possible) is based on clinical findings and special investigations (Table 1). The boundaries between groups are not fixed, and patients can interchange between groups, based on results of laboratory and radiological investigations. Definite TBM requires demonstration of acid-fast bacilli in the CSF, Mycobacterium tuberculosis culture from CSF, a positive nucleic acid amplification test (PCR) of CSF or histopathological evidence of Mycobacterium tuberculosis from a central nervous system site.

Patients must have a clinical diagnosis of meningitis including one or more of the following symptoms: headache, irritability, vomiting, fever and neck stiffness (Table 1). The diagnosis of probable or possible TBM is based on 1) clinical findings 2) CSF results 3) neuroimaging findings 4) evidence for TB outside the central nervous system and 5) additional laboratory criteria. A scoring system then determines whether a patient falls in the probable or possible TBM category (Table1). Points are allocated for a positive finding in each of the categories, with a maximum score for each category. A total score of at least 10 is compatible with probable TBM, while a total score of at least 6 equates with a possible TBM diagnosis. A certain number of points have to come from the CSF or neuro-imaging criteria to ensure that the patient is indeed suffering from meningitis and not some other chronic type of tuberculosis. It is also suggested by the consensus

group that all patients should have additional CSF investigations to exclude alternative causes for the clinical presentation.

In practice, a clinical case definition of bacterial meningitis is needed because most patients admitted to a tertiary setting such as Tygerberg Hospital, will already have had antibiotics, which usually results in negative blood and CSF cultures [13]. Similarly, a definite or clinical diagnosis of viral meningitis as possible cause for aseptic meningitis is critical, since aseptic meningitis is frequently caused by viruses [14]. If an alternative cause is found for the clinical presentation, a diagnosis of not-TBM should be made, excepting where dual infection is confirmed.

CSF diagnosis of TBM

It has been shown in adults that the bacterial yield of *Mycobacterium tuberculosis* can be improved by obtaining larger volumes of CSF and optimizing centrifugation [15, 16]. Additional measures for improving sensitivity in identification of the organism include, increasing the amount of time taken with microscopy and alternative staining methods [16]. Based on calculations of Den Hertog *et al*., optimal CSF centrifugation in case of mycobacterium tuberculosis infection should be at a minimum speed and duration of 3200x g for 22 minutes [17].

Acid-fast staining is the most rapid method for the detection of mycobacteria in clinical samples. Acid-fast staining methods include the Ziehl-Nielsen (ZN) and Auramine O stains. Since CSF is sterile, as a rule, the demonstration of acid-fast bacilli in the CSF is highly suggestive of TBM. Fluorescence microscopy uses a lower power objective lens than conventional microscopy. This enables a more rapid and cost-effective assessment of the full area of a slide. A disadvantage is the possibility of false-positive results due to inorganic objects incorporating the fluorochrome dyes [18]. Unfortunately, the use of fluorescence microscopy has been limited in resource-poor settings by the short half-life and high cost of the traditional mercury vapour lamp. Light-emitting diode (LED) technology provides a cost-efficient alternative; it is cheap, robust and has very low energy requirements [19].

In response to the need for more rapid diagnosis of TB and the identification of resistant strains, nucleic acid amplification tests specific for *Mtb* have been developed. In a comparative study

between the Genotype Mycobacteria direct assay, the Gen-Probe *Mycobacterium tuberculosis* amplified direct test and the Genotype MTBDRplus for direct detection of mycobacterium tuberculosis complex in clinical samples, the Genotype MTBDRplus had the highest sensitivity (97.9%) [20]. In a study evaluating the *Mtb complex* detection rate using the Genotype MTBDRplus assay on acid-fast bacillus (AFB) smear positive sputum and PCR positive non- sputum specimens, the sensitivities were 93.8% and 77.8% respectively [21]. For the Genotype MTBDRplus assay, the sensitivity (98.1%) and specificity (98.7%) for rifampicin resistance is very high. The accuracy for isoniazid is variable, with a lower sensitivity (84.3%) but very high specificity (99.5%) [22]. PCR is usually only done on culture. PCR on fresh CSF could potentially offer earlier confirmation of TBM diagnosis and resistance.

The GeneXpert MTB/RIF (Xpert) assay targets the rifampin resistance-associated *rpoB* gene region by heminested PCR with three specific primers and combines the sensitive detection of *M. tuberculosis* DNA and determination of rifampicin resistance using molecular beacons [23]. Processing time is short due to automation of bacterial lysis, DNA extraction, real-time PCR amplification, and amplicon detection in a single system. High sensitivity and specificity of the Xpert assay has been shown on sputa with the assay correctly identifying 77.3% of all culture-positive specimens [24]. Similar results are not applicable to extrapulmonary specimens, though, where the amount of mycobacterial DNA is much less. The test runs on the GeneXpert platform (Cepheid, Sunnyvale, CA) using a disposable plastic cartridge with all required reagents [25]. Although the WHO currently recommends Xpert as an initial diagnostic test for TBM, the sensitivity of the test on CSF samples is not as high as would be obtained with sputum samples and increased to 72% when CSF was centrifuged [8].

Use of host biomarkers in the diagnosis of TBM

Recent technological advances have made it possible to screen for many biomarkers in as little as 3 µl of sample using the Luminex multiplex cytokine beaded arrays. Using the Luminex platform, it is now possible to simultaneously detect the levels of 100 biomarkers in 3 to 50 µl (if using the Luminex/Bio-Plex 200 platform) and up to 500 markers if using the Luminex FlexMap 3D system. This platform has enabled the identification of novel host protein biomarkers for several diseases including TB. In previous studies, some of which were pioneered by our research group at

Stellenbosch University, novel biomarkers were detected in adult Quantiferon supernatants, which allowed for discrimination between TB disease and latent infection [26]. When the biomarkers identified in adults were investigated in children, a biosignature comprising of four markers diagnosed paediatric TB disease with an accuracy of 84% [27].

In a recently concluded large multi-centred pan-African study (the African European Tuberculosis Consortium –www.ae-tbc.eu), we observed that some of the markers that frequently showed potential in our previous TB-antigen stimulation studies[26-30] performed best either individually or in multi-marker models when measured in unstimulated supernatants. Further work on ex vivo serum biomarkers in 716 individuals that presented signs and symptoms suggestive of TB, prior to clinical diagnosis in five African countries (Namibia, Uganda, Malawi, The Gambia and South Africa), resulted in the identification of a seven-marker serum biosignature, which diagnosed TB disease in adults with high accuracy [31].

There are not many studies in literature that specifically employed multiplex cytokine detection platforms in the search of host biomarkers specifically for TBM. In a recently patented and published study conducted by our research group [32], we investigated the usefulness of some of the host markers that we identified in our previous studies as diagnostic candidates for TBM. A three-marker CSF biosignature comprising of IL-13, VEGF and cathelicidin LL-37, diagnosed TBM with a sensitivity of 52%, specificity of 95%, with positive and negative predictive values of 91% and 66% respectively [33]. In other biomarker studies, specifically investigating the use of whole blood RNA biosignatures in the diagnosis of TB disease, it was shown that host blood-based RNA transcripts might be useful in the diagnosis of active TB disease in both adults [34] and children [35]. Furthermore, a recent study identified blood RNA signature that showed potential as a biosignature for the risk of developing active TB disease [36]. However, none of these studies have been conducted specifically in children with TBM and it is not known, whether simple blood RNA signatures such as the ones already identified for pulmonary TB might be useful in this disease. The overall aim of the current study is therefore to assess the utility of ex vivo blood and CSF based host cytokine biomarkers and RNA transcripts as diagnostic tools for TBM. We would specifically be validating the diagnostic accuracy of our previously established 3-marker TBM CSF biosignature, evaluate other new host markers which have shown potential in adult studies

but which have not been investigated in children, in more children with TBM and also RNA signatures in blood samples collected from the same children. At the conclusion of the proposed study, the validated blood-based biosignatures identified in the present study shall be ready for possible incorporation into rapid, point-of-care diagnostic tools such as the one currently being developed for adult pulmonary TB by our research group (www.screen-tb.eu).

Hypothesis

CSF and blood samples obtained from children with TBM will possess distinct host biosignatures in comparison to CSF and blood samples from children with other forms of meningitis and these biosignatures can be used as a tool for the rapid diagnosis of TBM.

Aims

The main aims of the proposed project are as follows:

1) To validate a previously identified and patented three-marker CSF biosignature (IL-13, VEGF, cathelicidin LL37) for the diagnosis of TBM, in a new cohort of children with suspected TBM

2) To evaluate the usefulness of a recently identified and patented adult seven-marker serum protein biosignature (CRP, serum amyloid A, apolipoprotein A-1, transthyretin, complement factor H, interferon-gamma and IP-10) and other recently identified host markers, as a tool for the diagnosis of TBM

3) To investigate the usefulness of whole blood RNA transcripts as biosignatures for the diagnosis of TBM

4) To characterize the site of disease (CSF) versus peripheral blood immune profile of children with TBM, the response of immune cells to stimulation with *M.tb* antigens and explore differences between such responses in children with and those without TBM as part of student projects.

5) To develop a prototype point-of-care test based on host inflammatory protein biomarkers for

the rapid diagnosis of TBM (e.g. at the bedside) in children suspected of having TBM.

Patients and methods

1. Study design

A prospective study

2. Study setting

The study will be conducted at Tygerberg Children's Hospital, Parow Valley, Cape Town. The hospital is the tertiary academic hospital of the Faculty of Medicine and Health Sciences, University of Stellenbosch. Children with tuberculous meningitis are referred from primary care day hospitals, district and secondary level hospitals from our drainage area. Children with suspected TBM are referred to our institution to establish the diagnosis of TBM and to treat the complications associated with advanced disease (stage 2 and 3 TBM, e.g. hydrocephalus). Research samples collected for the purposes of the current study shall be processed at the Stellenbosch University Immunology Research Group (SUN-IRG) laboratory, Department of Biomedical Sciences, Faculty of Medicine and Health Sciences. The SUN-IRG laboratory is accredited by the South African National Accreditation Agency as a specialist Immunology Research laboratory.

3. Inclusion and exclusion criteria:

All children between the ages of 3 months and 13 years with suspected meningitis, and who require CSF examination for routine diagnostic purposes at Tygerberg Children's Hospital, where written consent is obtained will be included. If possible, assent will be obtained in those children older than 7 years who have a normal level of consciousness, i.e. a Glasgow Coma Score (GCS) of 15/15. Children 13 years and older will be excluded from the study. Failure to obtain written consent will also exclude children from the study.

The anticipated study period is November 2016 to December 2025. The estimated sample size, using a two-sample comparison of proportions (STATA®-Statistics/Data Analysis), required for each of the diagnostic aims of the study includes, at least, 72 children with definite or probable TBM and a total sample size of up to 460 patients (This includes children with TBM, bacterial

meningitis, viral meningitis and normal CSF). An average of 40 new TBM cases is admitted to our wards annually.

4. Diagnostic work-up

All patients will undergo a comprehensive clinical evaluation (Addendum1). Routine special investigations will be performed. CT of the brain will be done in all probable TBM cases and in other cases of meningitis when indicated. Air- encephalography will be performed in all TBM cases with hydrocephalus. MR imaging will be done as clinically indicated. Lumbar puncture will be performed and the following CSF investigations performed: appearance and colour determination; differential cell count determination by standard methods; protein, glucose and chloride determination by standard methods; centrifugation with Gram stain and India ink examination of the deposit; culture of the centrifuged deposit on blood agar plates for pyogenic bacteria; Auramine "O" staining & fluorescence microscopy, culture using the BBL™MGIT(Mycobacterium Growth Indicator Tubes)™ of Becton and Dickinson and detection of *Mtb* DNA using the HAIN Genotype MTBDRplus kit. Samples will be tested using Xpert Ultra and newer technologies e.g. AlereQ TB and the Savannah MTB assay. Microbiomic analysis will be performed on microbial DNA extracted from patient specimens and sequenced on the Illumina MiSeq platform. All data will be recorded on a pre-designed clinical data capture sheet (see addendum) and entered into a computerized database. Finally, data analysis will be carried out using appropriate software and statistical packages.

The diagnosis of TBM will be made based on the following criteria:

A diagnosis of **probable** TBM will be made if two or more of the following criteria are present in the setting of a characteristic history and CSF changes associated with TBM: A positive history of contact with an adult TB case, a positive tuberculin skin test, a chest x-ray suggestive of pulmonary tuberculosis (hilar lymphadenopathy, miliary tuberculosis or cavitation, CT or MRI demonstrating the characteristic features of TBM (ventricular dilatation, meningovascular enhancement and/or granuloma/s), poor weight gain or weight crossing percentiles documented on the Road to Health Card or positive identification of acid fast bacilli from gastric washings (microscopy or culture).

A diagnosis of **definite** TBM will be made if acid-fast bacilli is present in the CSF, *Mtb* is cultured

from CSF, a nucleic acid amplification test of CSF is positive or there is histopathological evidence of *Mtb* from another central nervous system site. The degree of TBM will be classified according to the proposed modified British Medical Research Council (BMRC) classification of 1948. -Stage I: Glasgow Coma Scale (GCS) of 15 and no focal neurology, Stage IIa: GCS of 15 plus focal neurology, Stage IIb: GCS of 11-14 with focal neurology, Stage III: GCS <11. All investigations required for establishing a diagnosis (TBM or no TBM) are usually done routinely and shall therefore not specifically be as part of the current study.

5. Treatment

All patients admitted with a diagnosis of TBM will be treated with standard regimen, four anti-tuberculous drugs for 6 months and prednisone (2mg/kg body weight; maximum dose 60mg daily) for the first month of treatment. All patients with ventriculomegaly on computed tomography will undergo an air encephalogram. Children with non-communicating hydrocephalus will undergo immediate ventriculoperitoneal (VP) shunting. Response to treatment will be monitored clinically and by a repeat CT scan of the brain after the first month of treatment.

All patients admitted with a diagnosis of bacterial meningitis will be treated with standard regimen third generation cephalosporin. Viral meningitis will be treated symptomatically; cases of suspected or proven herpes encephalitis will be treated with acyclovir. All HIV co-infected patients will be offered antiretroviral therapy (ART). Patients treated for TBM will only be started on ART after 4-6 weeks of anti-tuberculous therapy to minimize the risk of immune reconstitution inflammatory syndrome (IRIS). An exception will be made in cases who are severely immune-suppressed; they may be started on ART immediately. ART will consist of first-line therapy (for drugs &dosages per kg see addendum 2). Baseline CD4 counts and viral loads will be done before initiation of ART and repeated at 6 monthly intervals.

Collection and processing of samples for the proposed study

All samples required for the current study shall be collected during specimen collection for routine diagnosis. That is, specimens shall not specifically be collected from any study participant solely for the purposes of this study. During the collection of samples (CSF and whole blood) for routine clinical investigations, the alcohol swab used to sterilize the puncture site will be collected and

used to subtract background DNA found on the skin, during sequence analysis for the microbiome aspects of the study (aim 4). About 5 mL of CSF (3-7ml would be aimed for dependent on age and ease of lumbar puncture), shall be collected into sterile cryo tubes (Greiner bio-one) for use for research purposes, depending on how much is left over after specimens required for routine diagnosis have been collected. It must be stressed that although it is known that collection of up to 10ml of CSF in children with TBM may be beneficial in relieving of the pressure on the brain, hence help with alleviation of the symptoms, the amount of CSF collected will depend on the age, clinical presentation of the child, and whether collection of the specimen (3 to 7mL) will be beneficial to the child as determined by the specialist neurologist or medical practitioner treating the child. This will be followed by the collection of 1ml of whole blood into a serum blood tube and 2.5ml into a paxgene blood RNA tube, for the isolation of messenger RNA for future gene expression studies (aim 3). Furthermore, an additional 1 ml of blood will be collected from a subset of children for investigation of immune cells (whole blood intracellular staining by Flow cytometry; aim 7). Whether such an additional blood specimen is collected will depend on the judgement of the treating clinician and specialist paediatric neurologist, with the child's wellbeing always taking priority over the research. Where possible, mid-stream urine will be collected into sterile collection tubes for diagnostic analysis as part of aims 5 and 6. Therefore, depending on the judgement of the clinician and specialist paediatric neurologist, a total of up to 5mL (3 to 7ml) of extra CSF and up to 4.5ml of whole blood and up to 5 mL of urine (where possible) shall be collected from each consenting study participant for the purposes of this study. The proposed procedure (blood and CSF collection) was used in previously approved and now completed studies which provided samples for the pilot studies leading to this validation study (**HREC ref no**: **N11/01/006,** Principal Investigator, Dr Regan Solomons; and **Ref no: N09/10/265,** Principal Investigator, Dr D Visser)**.**

After collection of specimens for routine diagnostic purposes, the proposed research specimens shall be collected from consenting study participants and sent to the SUN Immunology Research laboratory, Fourth Floor, Education Building, where processing of these specimens shall occur. The CSF samples shall be centrifuged, aliquoted into 250µl amounts and frozen at minus 80 degrees until used for biomarker analysis using the Luminex technology, as was done in the pilot study (33). If more than 1mL of CSF is available, aliquots of the CSF will be used for immune cell

analysis by flow cytometry as mentioned above. Similarly, the 1ml of blood collected into a serum tube shall be centrifuged and serum harvested and stored at minus 80 degrees until used for biomarker analysis. The additional 1ml of blood, if collected, will also be used for flow cytometry as part of a student project. For the Paxgene blood, the tubes shall be frozen and when needed for use, RNA shall be isolated using the Paxgene blood RNA isolation kit. We will then use downstream techniques including quantitative Real Time PCR or other suitable technique to look at the usefulness of the RNA blood biosignatures already described in publications such as references (34-36), and also new signatures in the diagnosis of TBM. All sample processing shall be done according to standard laboratory procedures which are currently used in the SANAS accredited SUN-IRG laboratory.

Both the CSF and urine for metabolomics, up to 2ml and 5ml respectively, will be transported to the metabolic laboratory at Northwest University in Potchefstroom for analysis of metabolites. This will be done by performing mass spectroscopy, coupled with chromatography, followed by complex data analysis. The investigations at the metabolic laboratory will be performed and interpreted by Dr S Mason, from the biochemistry division at Northwest University. Stellenbosch University students will use aliquots of the CSF in future for unbiased evaluation of novel protein biomarkers that may be used in the diagnosis of TBM, for optimization of the test that will be developed as part of this project or biomarkers that may guide clinical patient management.

Statistical methods and analysis plan

Differences in the concentrations of individual host biomarkers between participants with TBM disease and those without TBM shall be evaluated by the Mann–Whitney U-test for non-parametric data analysis or the students t test, if data is normally distributed. P-values shall be deemed significant if less than 5%. The diagnostic accuracy of individual host biomarkers shall be investigated by receiver operator characteristics (ROC) curve analysis. The predictive abilities of combinations of analytes (biosignatures) shall be investigated by General discriminant analysis (GDA), random forests, and other biomarker analysis techniques, following the training/test set approach as done in previous studies [27, 31-33]. For Flow cytometry data, cell gating and data analysis will first be carried out on FlowJo software (Oregon, USA), followed by parametric (t-test) or non-parametric (Mann-Whitney U-) tests to assess the differences in quantities of immune cell

subpopulations, intra and extracellular cytokines and other biomarkers among children with TBM and those with other meningitis. Kruskall-Wallies test or one-way ANOVA with post-hoc analysis will be performed for comparison of more than 2 groups as advised by bioinformaticians from the Division of Molecular Biology and Human Genetics.

Ethical concerns and consent

Informed consent will be obtained from all parents whose children are entered into the study. Only patients, whose legal care-givers give written consent for the study will be included. The information and consent document will be explained in the 3 main languages spoken in our area and obtained in the home language of parents/legal guardians. All consent forms will be back translated into English, to ensure clarity and accuracy, in accordance with South African Good Clinical Practice guidelines. As mentioned above, assent will be obtained in those children older than 7 years who have a normal level of consciousness, i.e. a Glasgow Coma Score (GCS) of 15/15. Urine, CSF and blood samples shall be collected from consenting study participants only where possible, and as deemed possible by the treating clinician and specialist paediatric neurologists. It is important to note that about 10ml of CSF may be collected from children diagnosed with TBM and collection of the CSF helps relieve the pressure on the brain caused by the fluid, thus alleviating symptoms. Ethical clearance for this study was already obtained from the Health Research Ethics Committee of the Faculty of Medicine and Health Sciences, University of Stellenbosch (Project Reference #: 4130 and HREC Reference #: N16/11/142). However, we are hereby requesting an amendment to the ethics to indicate that the project is now funded by the European and Developing Countries Clinical Trials Partnership (EDCTP), who have provided funds for us to validate the biomarkers, followed by development of a rapid point-of-care diagnostic test that may assist in the early management of TBM in children with the assistance of the Engineering Faculty at Stellenbosch University (Prof Willem Perold and students). The Engineers have developed a promising prototype platform to which validated biomarkers from this project shall be incorporated, followed by evaluation of the usefulness of the test during the course of the project. These later aspects (test development and evaluation) were not included in the version of the proposal that was previously approved by the HREC.

All information/data collected on the patients will remain confidential. All information/data

collected will be kept in a separate password protected file and this will only be known to and accessed by the Clinical Principal Investigator (Prof R Solomons). All samples sent to the Immunology Research Laboratory shall be anonymized and only identifiable by RedCap generated bar codes. Patients/legal guardians shall be informed that their refusal to participate in the present study shall not influence their care in any way. Good clinical practice guidelines will be adhered to.

Funding

Most of the special investigations are part of the routine workup of TBM, bacterial and viral meningitis patients at Tygerberg Children's Hospital. The costs for the extra tubes for collection of the additional specimens for research purposes and for all the research experiments including biomarker validation studies, development and evaluation of the point-of-care test shall be funded by an EDCTP (European and Developing Countries Clinical Trials Partnership) Senior Fellowship that was recently awarded. The project has been funded for a period of five years.

References

1.Donald PR, Cotton MF, Hendricks MK, Schaaf HS, de Villiers JN, Willemse TE. Pediatric meningitis in the Western Cape Province of South Africa. J Trop Pediatr. 1996;42(5):256-61. Epub 1996/10/01. PubMed PMID: 8936954.

2.World Health Organisation. Global Tuberculosis Control; Epidemiology, Strategy and Financing. World Health Organisation Report 2009. 2009 2009. Report No.

3.World Health Organisation. The End TB Strategy. 2015.

4.Schoeman J, Wait J, Burger M, van Zyl F, Fertig G, van Rensburg AJ, Springer P, Donald P. Long-term follow up of childhood tuberculous meningitis. Dev Med Child Neurol. 2002;44(8):522-6. Epub 2002/09/11. PubMed PMID: 12206617.

5.Thwaites GE, van Toorn R, Schoeman J. Tuberculous meningitis: more questions, still too few answers. Lancet Neurol. 2013;12(10):999-1010. Epub 2013/08/27. doi: 10.1016/s1474-4422(13)70168-6. PubMed PMID: 23972913.

6.Hosoglu S, Geyik MF, Balik I, Aygen B, Erol S, Aygencel TG, Mert A, Saltoglu N, Dokmetas I, Felek S, Sunbul M, Irmak H, Aydin K, Kokoglu OF, Ucmak H, Altindis M, Loeb M. Predictors of outcome in patients with tuberculous meningitis. Int J Tuberc Lung Dis. 2002;6(1):64-70. Epub 2002/04/05. PubMed PMID: 11931403.

7.van Well GT, Paes BF, Terwee CB, Springer P, Roord JJ, Donald PR, van Furth AM, Schoeman JF. Twenty years of pediatric tuberculous meningitis: a retrospective cohort study in the western cape of South Africa. Pediatrics. 2009;123(1):e1-8. Epub 2009/04/16. PubMed PMID: 19367678.

8.Bahr NC, Marais S, Caws M, van Crevel R, Wilkinson RJ, Tyagi JS, Thwaites GE, Boulware DR. GeneXpert MTB/Rif to Diagnose Tuberculous Meningitis: Perhaps the First Test but not the Last. Clin Infect Dis.

2016;62(9):1133-5. Epub 2016/03/12. doi: 10.1093/cid/ciw083. PubMed PMID: 26966284; PMCID: PMC4826457.
9.Bhigjee AI, Padayachee R, Paruk H, Hallwirth-Pillay KD, Marais S, Connoly C. Diagnosis of tuberculous meningitis: clinical and laboratory parameters. Int J Infect Dis. 2007;11(4):348-54. Epub 2007/02/27. doi: 10.1016/j.ijid.2006.07.007. PubMed PMID: 17321183.
10.Andronikou S, Wilmshurst J, Hatherill M, VanToorn R. Distribution of brain infarction in children with tuberculous meningitis and correlation with outcome score at 6 months. Pediatr Radiol. 2006;36(12):1289-94. Epub 2006/10/13. doi: 10.1007/s00247-006-0319-7. PubMed PMID: 17031634.
11.Saitoh A, Pong A, Waecker NJ, Jr., Leake JA, Nespeca MP, Bradley JS. Prediction of neurologic sequelae in childhood tuberculous meningitis: a review of 20 cases and proposal of a novel scoring system. Pediatr Infect Dis J. 2005;24(3):207-12. Epub 2005/03/08. PubMed PMID: 15750455.
12.Marais S, Thwaites G, Schoeman JF, Torok ME, Misra UK, Prasad K, Donald PR, Wilkinson RJ, Marais BJ. Tuberculous meningitis: a uniform case definition for use in clinical research. Lancet Infect Dis. 2010;10(11):803-12. Epub 2010/09/09. doi: 10.1016/s1473-3099(10)70138-9. PubMed PMID: 20822958.
13.WHO. Recommended surveillance standards for surveillance of selected vaccine-preventable diseases. 2003.
14.Bottner A, Daneschnejad S, Handrick W, Schuster V, Liebert UG, Kiess W. A season of aseptic meningitis in Germany: epidemiologic, clinical and diagnostic aspects. Pediatr Infect Dis J. 2002;21(12):1126-32. Epub 2002/12/19. doi: 10.1097/01.inf.0000040713.64184.ad. PubMed PMID: 12488662.
15.Case definitions for infectious conditions under public health surveillance. Centers for Disease Control and Prevention. MMWR Recomm Rep. 1997;46(Rr-10):1-55. Epub 1997/05/02. PubMed PMID: 9148133.
16.Thwaites GE, Chau TT, Farrar JJ. Improving the bacteriological diagnosis of tuberculous meningitis. J Clin Microbiol. 2004;42(1):378-9. Epub 2004/01/13. PubMed PMID: 14715783; PMCID: PMC321694.
17.den Hertog AL, Klatser PR, Anthony RM. Buoyant density of Mycobacterium tuberculosis: implications for sputum processing. Int J Tuberc Lung Dis. 2009;13(4):466-71. Epub 2009/04/02. PubMed PMID: 19335952.
18.Steingart KR, Henry M, Ng V, Hopewell PC, Ramsay A, Cunningham J, Urbanczik R, Perkins M, Aziz MA, Pai M. Fluorescence versus conventional sputum smear microscopy for tuberculosis: a systematic review. Lancet Infect Dis. 2006;6(9):570-81. Epub 2006/08/26. doi: 10.1016/s1473-3099(06)70578-3. PubMed PMID: 16931408.
19.Marais BJ, Brittle W, Painczyk K, Hesseling AC, Beyers N, Wasserman E, van Soolingen D, Warren RM. Use of light-emitting diode fluorescence microscopy to detect acid-fast bacilli in sputum. Clin Infect Dis. 2008;47(2):203-7. Epub 2008/06/06. doi: 10.1086/589248. PubMed PMID: 18532893.
20.Neonakis IK, Gitti Z, Baritaki S, Petinaki E, Baritaki M, Spandidos DA. Evaluation of GenoType mycobacteria direct assay in comparison with Gen-Probe Mycobacterium tuberculosis amplified direct test and GenoType MTBDRplus for direct detection of Mycobacterium tuberculosis complex in clinical samples. J Clin Microbiol. 2009;47(8):2601-3. Epub 2009/06/26. doi: 10.1128/jcm.02351-08. PubMed PMID: 19553580; PMCID: PMC2725688.
21.Zhang L, Ye Y, Duo L, Wang T, Song X, Lu X, Ying B, Wang L. Application of genotype MTBDRplus in rapid detection of the Mycobacterium tuberculosis complex as well as its resistance to isoniazid and rifampin in a high volume laboratory in Southern China. Mol Biol Rep. 2011;38(3):2185-92. Epub 2010/09/21. doi: 10.1007/s11033-010-0347-0. PubMed PMID: 20852939.
22.Ling DI, Zwerling AA, Pai M. GenoType MTBDR assays for the diagnosis of multidrug-resistant tuberculosis: a meta-analysis. Eur Respir J. 2008;32(5):1165-74. Epub 2008/07/11. doi: 10.1183/09031936.00061808. PubMed PMID: 18614561.
23.El-Hajj HH, Marras SA, Tyagi S, Kramer FR, Alland D. Detection of rifampin resistance in

Mycobacterium tuberculosis in a single tube with molecular beacons. J Clin Microbiol. 2001;39(11):4131-7. Epub 2001/10/30. doi: 10.1128/jcm.39.11.4131-4137.2001. PubMed PMID: 11682541; PMCID: PMC88498.
24.Hillemann D, Rusch-Gerdes S, Boehme C, Richter E. Rapid molecular detection of extrapulmonary tuberculosis by the automated GeneXpert MTB/RIF system. J Clin Microbiol. 2011;49(4):1202-5. Epub 2011/01/29. doi: 10.1128/jcm.02268-10. PubMed PMID: 21270230; PMCID: PMC3122824.
25.Raja S, Ching J, Xi L, Hughes SJ, Chang R, Wong W, McMillan W, Gooding WE, McCarty KS, Jr., Chestney M, Luketich JD, Godfrey TE. Technology for automated, rapid, and quantitative PCR or reverse transcription-PCR clinical testing. Clin Chem. 2005;51(5):882-90. Epub 2005/03/05. doi: 10.1373/clinchem.2004.046474. PubMed PMID: 15746302.
26.Chegou NN, Black GF, Kidd M, van Helden PD, Walzl G. Host markers in QuantiFERON supernatants differentiate active TB from latent TB infection: preliminary report. BMC Pulm Med. 2009;9:21. Epub 2009/05/19. doi: 10.1186/1471-2466-9-21. PubMed PMID: 19445695; PMCID: PMC2696407.
27.Chegou NN, Detjen AK, Thiart L, Walters E, Mandalakas AM, Hesseling AC, Walzl G. Utility of host markers detected in Quantiferon supernatants for the diagnosis of tuberculosis in children in a high-burden setting. PLoS One. 2013;8(5):e64226. Epub 2013/05/22. doi: 10.1371/journal.pone.0064226. PubMed PMID: 23691173; PMCID: PMC3655018.
28.Awoniyi DO, Teuchert A, Sutherland JS, Mayanja-Kizza H, Howe R, Mihret A, Loxton AG, Sheehama J, Kassa D, Crampin AC, Dockrell HM, Kidd M, Rosenkrands I, Geluk A, Ottenhoff TH, Corstjens PL, Chegou NN, Walzl G. Evaluation of cytokine responses against novel Mtb antigens as diagnostic markers for TB disease. J Infect. 2016. Epub 2016/06/18. doi: 10.1016/j.jinf.2016.04.036. PubMed PMID: 27311746.
29.Chegou NN, Essone PN, Loxton AG, Stanley K, Black GF, van der Spuy GD, van Helden PD, Franken KL, Parida SK, Klein MR, Kaufmann SH, Ottenhoff TH, Walzl G. Potential of host markers produced by infection phase-dependent antigen-stimulated cells for the diagnosis of tuberculosis in a highly endemic area. PLoS One. 2012;7(6):e38501. Epub 2012/06/14. doi: 10.1371/journal.pone.0038501. PubMed PMID: 22693640; PMCID: PMC3367928.
30.Essone PN, Chegou NN, Loxton AG, Stanley K, Kriel M, van der Spuy G, Franken KL, Ottenhoff TH, Walzl G. Host cytokine responses induced after overnight stimulation with novel M. tuberculosis infection phase-dependent antigens show promise as diagnostic candidates for TB disease. PLoS One. 2014;9(7):e102584. Epub 2014/07/16. doi: 10.1371/journal.pone.0102584. PubMed PMID: 25025278; PMCID: PMC4099213.
31.Chegou NN, Sutherland JS, Malherbe S, Crampin AC, Corstjens PL, Geluk A, Mayanja-Kizza H, Loxton AG, van der Spuy G, Stanley K, Kotze LA, van der Vyver M, Rosenkrands I, Kidd M, van Helden PD, Dockrell HM, Ottenhoff TH, Kaufmann SH, Walzl G. Diagnostic performance of a seven-marker serum protein biosignature for the diagnosis of active TB disease in African primary healthcare clinic attendees with signs and symptoms suggestive of TB. Thorax. 2016;71(9):785-94. Epub 2016/05/06. doi: 10.1136/thoraxjnl-2015-207999. PubMed PMID: 27146200.
32.Jacobs R, Malherbe S, Loxton AG, Stanley K, van der Spuy G, Walzl G, Chegou NN. Identification of novel host biomarkers in plasma as candidates for the immunodiagnosis of tuberculosis disease and monitoring of tuberculosis treatment response. Oncotarget. 2016;7(36):57581-92. Epub 2016/08/25. doi: 10.18632/oncotarget.11420. PubMed PMID: 27557501.
33.Visser DH, Solomons RS, Ronacher K, van Well GT, Heymans MW, Walzl G, Chegou NN, Schoeman JF, van Furth AM. Host immune response to tuberculous meningitis. Clin Infect Dis. 2015;60(2):177-87. Epub 2014/10/11. doi: 10.1093/cid/ciu781. PubMed PMID: 25301213.
34.Berry MP, Graham CM, McNab FW, Xu Z, Bloch SA, Oni T, Wilkinson KA, Banchereau R, Skinner J, Wilkinson RJ, Quinn C, Blankenship D, Dhawan R, Cush JJ, Mejias A, Ramilo O, Kon OM, Pascual V, Banchereau J, Chaussabel D, O'Garra A. An interferon-inducible neutrophil-driven blood transcriptional signature in human tuberculosis. Nature. 2010;466(7309):973-7. Epub 2010/08/21. doi:

10.1038/nature09247. PubMed PMID: 20725040; PMCID: PMC3492754.
35.Anderson ST, Kaforou M, Brent AJ, Wright VJ, Banwell CM, Chagaluka G, Crampin AC, Dockrell HM, French N, Hamilton MS, Hibberd ML, Kern F, Langford PR, Ling L, Mlotha R, Ottenhoff TH, Pienaar S, Pillay V, Scott JA, Twahir H, Wilkinson RJ, Coin LJ, Heyderman RS, Levin M, Eley B. Diagnosis of childhood tuberculosis and host RNA expression in Africa. N Engl J Med. 2014;370(18):1712-23. Epub 2014/05/03. doi: 10.1056/NEJMoa1303657. PubMed PMID: 24785206; PMCID: PMC4069985.
36.Zak DE, Penn-Nicholson A, Scriba TJ, Thompson E, Suliman S, Amon LM, Mahomed H, Erasmus M, Whatney W, Hussey GD, Abrahams D, Kafaar F, Hawkridge T, Verver S, Hughes EJ, Ota M, Sutherland J, Howe R, Dockrell HM, Boom WH, Thiel B, Ottenhoff TH, Mayanja-Kizza H, Crampin AC, Downing K, Hatherill M, Valvo J, Shankar S, Parida SK, Kaufmann SH, Walzl G, Aderem A, Hanekom WA. A blood RNA signature for tuberculosis disease risk: a prospective cohort study. Lancet. 2016;387(10035):2312-22. Epub 2016/03/28. doi: 10.1016/s0140-6736(15)01316-1. PubMed PMID: 27017310.